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THE SIMULTANEOUS RELEASE OF HISTAMINE AND A HISTAMINE-DESTROYING FACTOR DURING ANAPHYLAXIS IN RATS

BY C. F. CODE, D. T. CODY, MARGARET HURN, J. C. KENNEDY AND MARTHA J. STRICKLAND

From the Mayo Clinic and Mayo Foundation, Rochester, Minnesota, U.S.A.

(*Received* 8 *June* 1960)

Mota (1957, 1958) has demonstrated that the concentration of histamine in the plasma of rats is increased during anaphylactic shock. He used alum-precipitated horse serum or *Bordetella* (*haemophilus*) *pertussis* vaccine as adjuvants to aid the development of the supersensitivity. Maruno (1958*b*) has shown that the concentration of histamine in the whole blood of rats previously injected with horse serum alone is greater after challenge with the serum than in control unsensitized animals. The objectives of this study were, first, to confirm the release of histamine during anaphylactic shock in pertussis-vaccinated rats; secondly, to explore the possibility of a simultaneous release of a histamine-destroying factor in such rats as has been shown already for rabbits by Rose & Leger (1952); and, finally, to determine whether release of histamine and a histamine-destroying factor occurred in anaphylactic shock in rats rendered susceptible to sensitization by adrenalectomy instead of by the use of adjuvants.

METHODS

Healthy white male rats of the Sprague-Dawley strain, weighing 150–250 g, were used in all experiments. The intact animals were sensitized by a single intraperitoneal injection of 25 mg crystalline ovalbumin dissolved in 1 ml. NaCl solution, 0·9 g/100 ml. This was combined in the syringe for injection with 0·5 ml. of a saline suspension of phase-1 *Bordetella* (*haemophilus*) *pertussis* vaccine containing approximately 30×10^9 killed pertussis organisms. The adrenalectomized rats were sensitized by the subcutaneous injection of 0·5 ml. of sterile horse serum given on four consecutive days without the addition of pertussis vaccine. Rats sensitized to ovalbumin were challenged by the intravenous injection of 3–7 mg of the crystalline protein dissolved in 0·25–0·8 ml. of 0·9 % NaCl solution. One millilitre of the undiluted horse serum was used as the challenging intravenous injection for the adrenalectomized animals.

Anaesthesia was induced by the inhalation of ether or by intraperitoneal injection of pentobarbital sodium 4 mg/100 g body weight. The adrenal glands were removed through a single dorsal mid line incision at the level of the kidneys. The glands were approached

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ERRATUM

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p. 504, last two lines: *for* 'cholesterol' *read* 'sterol'.

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THE SIMULTANEOUS RELEASE OF HISTAMINE AND A HISTAMINE-DESTROYING FACTOR DURING ANAPHYLAXIS IN RATS

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(*Received* 8 *June* 1960)

Mota (1957, 1958) has demonstrated that the concentration of histamine in the plasma of rats is increased during anaphylactic shock. He used alum-precipitated horse serum or *Bordetella* (*haemophilus*) *pertussis* vaccine as adjuvants to aid the development of the supersensitivity. Maruno (1958*b*) has shown that the concentration of histamine in the whole blood of rats previously injected with horse serum alone is greater after challenge with the serum than in control unsensitized animals. The objectives of this study were, first, to confirm the release of histamine during anaphylactic shock in pertussis-vaccinated rats; secondly, to explore the possibility of a simultaneous release of a histamine-destroying factor in such rats as has been shown already for rabbits by Rose & Leger (1952); and, finally, to determine whether release of histamine and a histamine-destroying factor occurred in anaphylactic shock in rats rendered susceptible to sensitization by adrenalectomy instead of by the use of adjuvants.

METHODS

Healthy white male rats of the Sprague-Dawley strain, weighing 150–250 g, were used in all experiments. The intact animals were sensitized by a single intraperitoneal injection of 25 mg crystalline ovalbumin dissolved in 1 ml. NaCl solution, 0·9 g/100 ml. This was combined in the syringe for injection with 0·5 ml. of a saline suspension of phase-1 *Bordetella* (*haemophilus*) *pertussis* vaccine containing approximately 30×10^9 killed pertussis organisms. The adrenalectomized rats were sensitized by the subcutaneous injection of 0·5 ml. of sterile horse serum given on four consecutive days without the addition of pertussis vaccine. Rats sensitized to ovalbumin were challenged by the intravenous injection of 3–7 mg of the crystalline protein dissolved in 0·25–0·8 ml. of 0·9 % NaCl solution. One millilitre of the undiluted horse serum was used as the challenging intravenous injection for the adrenalectomized animals.

Anaesthesia was induced by the inhalation of ether or by intraperitoneal injection of pentobarbital sodium 4 mg/100 g body weight. The adrenal glands were removed through a single dorsal mid line incision at the level of the kidneys. The glands were approached

through small incisions just below the margin of the ribs on each side, care being taken to remove each gland without rupture of its capsule. After operation the rats were given 2% glucose in 0·9% NaCl solution to drink.

In the initial phases of the study blood was withdrawn, before and after shock, by cardiac puncture under ether anaesthesia. Later, suspicion was aroused that the trauma associated with cardiac puncture was contributing to the variability of the results. Blood was therefore subsequently withdrawn, under pentobarbital sodium anaesthesia, through a polyethylene catheter placed in the superior vena cava just above the heart by threading it down the jugular vein through a small incision in the neck. In the first and largest group of animals studied blood was withdrawn by both techniques. In the later tests and all those performed on adrenalectomized rats blood was taken via the catheter.

The concentration of histamine in the blood and plasma was determined by a modification of the Barsoum & Gaddum (1935) method (Code & McIntire, 1956) and is expressed in terms of the base throughout. When testing for the destruction of histamine by blood, one sample of blood was extracted immediately after collection and the remainder was set aside for varying periods either at room temperature or at 37–38° C. On some occasions oxygen was passed through the vessel containing the plasma or blood for a brief period before incubation. In some instances histamine was added to the blood before incubation. Occasionally the concentration of histamine in the plasma was determined without extraction by direct addition of the diluted plasma to the segment of guinea-pig ileum used in the assay.

RESULTS

Control experiments on intact and adrenalectomized rats

The mean concentration of histamine in the blood of sensitized but unchallenged animals was not significantly different from that of rats which had not received sensitizing injections; nor was there any significant difference in blood histamine between animals anaesthetized with ether and those anaesthetized with sodium pentobarbital. The values obtained in the 50 samples of blood withdrawn by both cardiac puncture and venous catheterization from the 50 rats under control conditions in the initial series were combined and gave a mean histamine concentration of $0{\cdot}136 \pm 0{\cdot}010$ μg/ml. Blood drawn by venous catheterization only, in the later series, gave a much lower mean of $0{\cdot}078 \pm 0{\cdot}008$ μg histamine/ml. Trauma during cardiac puncture was the likely reason for this difference, for when blood was drawn by cardiac puncture from four rats, first without and then with trauma, the values obtained with trauma were always greater. Likewise, when blood was taken by cardiac puncture or by venous catheterization from alternate rats of similar age, all samples of blood taken by cardiac puncture contained more histamine ($0{\cdot}240 \pm 0{\cdot}034$ μg/ml.) than those withdrawn through the catheter ($0{\cdot}078 \pm 0{\cdot}008$ μg/ml.). The mean concentration of histamine in the whole blood of 26 adrenalectomized rats was 0·09 μg/ml., which was not significantly different from blood of intact rats, withdrawn by the same technique (venous catheterization).

Anaphylactic shock

The concentration of histamine in the blood of intact rats during anaphylactic shock was always greater than that encountered under control conditions (Table 1). During anaphylactic shock the concentration of histamine in blood drawn by cardiac puncture from rats under ether anaesthesia was significantly less than the concentration in blood obtained by venous catheterization from rats under pentothal sodium anaesthesia (Table 1), probably because considerably more time was required to withdraw the blood by cardiac puncture than via the venous catheter.

TABLE 1. Histamine concentration in whole blood of rats during anaphylactic shock

Conditions	Sensitizing agent	Number of rats	Mean blood histamine (μg/ml.)
Intact rats			
Ether anaesthesia (cardiac puncture)	Ovalbumin	10	$0{\cdot}733 \pm 0{\cdot}160$
Pentobarbital anaesthesia (venous catheterization)	Ovalbumin	10	$1{\cdot}950 \pm 0{\cdot}193$
Adrenalectomized rats	Horse serum	20	$0{\cdot}680 \pm 0{\cdot}082$

Serial withdrawal of blood samples through the catheter allowed determination of the sequence of the changes in the concentration of histamine in the blood. In the control tests shown in Fig. 1 significant changes did not occur in the concentration of histamine in the blood of unsensitized rats after challenge by egg albumin or in the concentration of histamine in the blood of sensitized rats after challenge by 0·9 % NaCl solution. However, when sensitized rats were challenged with the specific antigen, a decisive increase in the concentration of histamine in the blood always developed with maximal concentrations usually occurring in samples withdrawn from 3–5 min after the challenge (Fig. 1). As is shown in Table 1, the concentration of histamine in the whole blood of adrenalectomized rats during anaphylactic shock ($0{\cdot}680 \pm 0{\cdot}082$ μg/ml.) always exceeded the range of values of blood drawn from similar rats under control conditions (0·09 μg/ml.).

Distribution of histamine in the blood

Blood was withdrawn by venous catheterization from a group of sensitized intact rats before and after challenge either with 0·9 % NaCl solution or with the specific antigen. The formed elements of the blood were then separated by centrifugation. In the initial tests centrifugation was done at room temperature. In later experiments the blood was promptly chilled and centrifuged at 0° C. The histamine content of some of the

plasma was tested immediately by adding it directly to the guinea-pig ileum. Usually considerable dilution was required for satisfactory assay. The remainder was subjected to chemical extraction and later assayed for histamine. Small quantities of free histamine, usually < 0·05 μg/ml., were identified regularly in the unextracted plasma of the control rats given 0·9% NaCl solution and somewhat smaller quantities were identified in extracts of such plasma (Table 2). Plasma from blood which had been promptly chilled and centrifuged at 0° C. after its withdrawal from animals in anaphylactic shock always contained a high concentration of histamine activity. The mean concentration in the plasma of seven rats was 2·36 μg/ml. by direct assay and 1·92 μg/ml. after extraction. Since only small

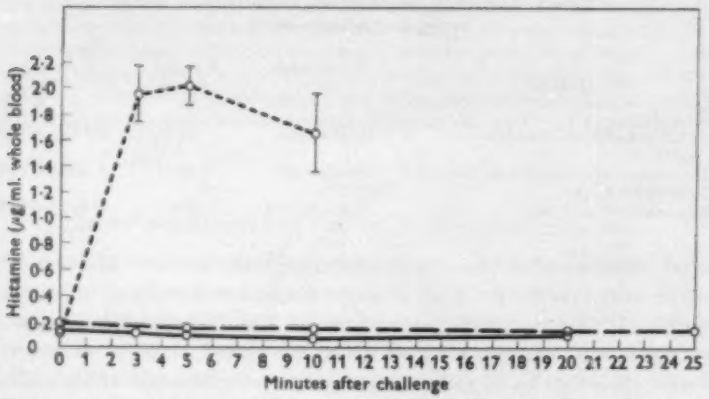

Fig. 1. The concentration of histamine in the blood of sensitized and unsensitized rats after challenge by intravenous injection of a solution of antigen or of 0·9% NaCl solution; blood withdrawn by venous catheter. —— Sensitized, saline-challenged; – – – unsensitized, antigen-challenged; - - - sensitized, antigen-challenged. Vertical bars show 2 × S.E. of mean.

quantities of plasma were available for extraction, some of the difference may be due to losses sustained during the manipulations involved in the process of chemical extraction.

The distribution of histamine between the plasma and the formed elements was determined in pooled samples of blood drawn under control conditions and during anaphylactic shock. In eight such control collections the mean distribution of the histamine in the blood was 13% in plasma, 66% in the white cell layer and 21% in the red cells. Losses of histamine during the separation were usually less than 20%. When similar collections of blood from rats in anaphylactic shock were tested, losses of 30–60% occurred and the plasma, instead of being rich in histamine, often contained no more than samples taken under control conditions. Since such losses

could not be ascribed to the procedures of separation or extraction, suspicion was aroused that an actual destruction of histamine was occurring.

Release of a histamine-destroying factor during anaphylactic shock

Blood drawn from animals in anaphylactic shock and allowed to stand undisturbed for 3 hr at room temperature consistently showed a significant (41 %) loss of histamine (Table 3). Control samples drawn from unshocked rats showed little or no destruction of histamine, even when sufficient histamine was added to bring the concentration within the range of that found in the blood in rats during anaphylactic shock (Table 3). Incubating the blood at 37° C increased the destruction of histamine to 54 % in 1·5 hr

TABLE 2. Histamine concentration in plasma of sensitized intact rats

Conditions	Number of rats	Mean plasma histamine (μg/ml.) Direct assay	Assay after extraction	Difference (%)
0·9 % NaCl injected	6	0·046 ± 0·004	0·032 ± 0·003	30
Albumin injected	7	2·360 ± 0·175	1·916 ± 0·183	19

TABLE 3. Change in histamine content of blood of rats during incubation

Number of tests	Condition of animal	Incubation of blood: Temperature (° C)	Incubation of blood: Hours	Histamine added to blood before incubation (μg/ml.)	Change in histamine content after incubation (%)
4	Unsensitized	R	3	2	+ 7
4	Sensitized	R	3	2	− 9
5	Shocked	R	3	Nil	−41
7	Unsensitized	37	1·5	2	− 5
4	Sensitized	37	1·5	2	− 3
9	Shocked	37	1·5	Nil	−54

R = room temperature.

in the blood from animals in anaphylactic shock, but did not increase the tiny quantities disappearing from blood drawn under control conditions. When the plasma of blood from rats in anaphylactic shock was separated and then incubated, the histamine in it was also quickly destroyed, indicating that part or all of the histamine-destroying factor found in the whole blood was present in the plasma fraction.

The results showed that during anaphylaxis in the rat a factor was released which destroyed the histamine liberated during the reaction. The question arose whether histamine added to the blood *in vitro* would be destroyed as effectively. To answer the question blood was drawn from a series of rats in anaphylactic shock and pooled. Samples for histamine determination were taken immediately and the remainder was incubated

at 37° C. for 1·5–3 hr, when further samples were withdrawn for histamine determination. Histamine was then added to the already incubated blood in amounts estimated to bring the concentration to approximately 2–3 μg/ml. and the blood was re-incubated for 1·5–3 hr. The results, which are given in Table 4, demonstrated clearly that the histamine-destroying factor in the blood was equally effective in the destruction of histamine added *in vitro* as it had been in destroying the histamine produced *in vivo* during the anaphylactic shock.

TABLE 4. Destruction of histamine in blood drawn from rats in anaphylactic shock

		Before addition of histamine		After addition of histamine*		
					Destroyed after reincubation (%)	
Pool number	Number of rats	Blood histamine before incubation (μg/ml.)	Destroyed after incubation† (%)	Blood histamine before reincubation (μg/ml.)	Without amino-guanidine	With amino-guanidine
1	2	0·83	82	1·88	68	—
2	2	1·60	87	1·92	82	Nil
3	4	1·65	—	3·45	50	4
4	2	—	—	2·92	44	—
5	1	—	—	3·30	10	—
6	2	1·25	20	3·00	11	Nil
7	3	1·35	78	2·00	68	Nil

* Histamine was added to the blood after incubation.
† Incubation at 37° C for 1·5–3 hr.

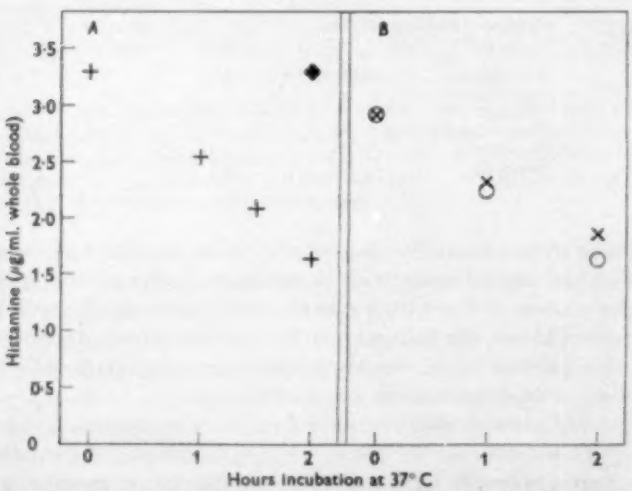

Fig. 2. Destruction *in vitro* of histamine in blood from rats in anaphylactic shock (see text). +, Shock blood + histamine; ◆, aminoguanidine added; ×, shock blood + control blood (1:1) + histamine; ○, shock blood + Tyrode soln. (1:1) + histamine.

Estimates were made next of the rate at which histamine was destroyed in blood drawn from rats in anaphylactic shock. Blood was taken from four rats in anaphylactic shock and pooled. Histamine was added in quantities calculated to bring the total concentration in the pool to between 3 and 3·5 μg/ml. After withdrawal of control samples, the pooled blood was incubated and samples were withdrawn at 1, 1½, and 2 hr. As is shown in Fig. 2*A*, destruction proceeded in a linear fashion during this period.

Since little or no destruction had occurred in the blood drawn from animals under control conditions, the prospect arose that the blood of healthy rats contained a factor which inhibited the histamine-destroying system. To test this possibility another pooled sample of blood from rats in anaphylactic shock was collected and to one portion of it blood from intact control rats was added, and to another an equal portion of Tyrode solution. The rate of destruction in the two portions of blood was almost identical (Fig. 2*B*). Thus, blood drawn from healthy rats does not contain an inhibitor of the histamine-destroying factor.

Destruction of histamine was usually demonstrable in blood drawn from adrenalectomized rats during anaphylactic shock, but the amounts destroyed during 1·5 hr incubation were generally less than those destroyed in blood from intact rats in anaphylactic shock. For example, in 17 samples of anaphylactic blood from adrenalectomized animals the mean destruction was 32%, whereas in similar tests on blood from intact rats in anaphylactic shock it was 48%.

The addition of sufficient aminoguanidine to the blood from animals in anaphylactic shock to produce a concentration of 0·4–0·5 μg/ml. of blood always completely prevented the destruction of histamine (Table 4; Fig. 2*A*). Since aminoguanidine is known to be an inhibitor of diamine oxidase (Zeller, 1938; Schuler, 1952) our results suggest that this enzyme is responsible for the histaminolytic power of anaphylactic blood.

DISCUSSION

The mean concentration of histamine in the whole blood of intact male rats which we studied first was 0·136 μg/ml. when blood was withdrawn by both cardiac puncture and venous catheter. This value is greater than the 0·035 μg/ml. found by Rose (1938) in male rats but less than the 0·63 μg/ml. reported by Maruno (1958*a*). In our later series, when blood was taken by venous catheter, a mean value of 0·078 μg/ml. was obtained. This is closer to the values of Rose, who took blood from the inferior vena cava without entering the thorax. Maruno obtained his samples by cardiac puncture. The differences in concentration therefore may be ascribed to differences in technique.

The plasma concentrations of 0·032 and 0·028 μg/ml. of unchallenged

rats found by us were less than the 0·186 μg/ml. reported in intact rats by Emmelin (1945; cardiac puncture) but comparable to the 0·035 μg/ml. reported by Halpern & Briot (1954; blood from retro-orbital plexus). Once again it seems most likely that the differences were due to differences in the technique employed for obtaining the blood.

Mota (1957, 1958) found that plasma from rats in anaphylactic shock contained increased quantities of histamine activity. He tested the plasma for histamine activity by direct addition to an isolated segment of guinea-pig ileum. Our results confirm his findings. The fact that the active substance survived the chemical extraction employed in this study supports the contention that it is histamine.

The exact role of histamine in the production of the symptoms of anaphylactic shock in rats is not clear. Mota has found, for example, that rats in which the stores of histamine had been depleted by 48/80 show minimal anaphylactic reactions. Sanyal & West (1958*b*), on the other hand, observed that rats in which the histamine stores were depleted by polymyxin B or by whole-body X-ray irradiation showed severe grades of anaphylaxis. The difference may have been associated with the mode of sensitization, for alum-precipitated protein was used as an adjuvant by Mota and *B. pertussis* by Sanyal & West. In addition, depletion of histamine stores is not likely to be the only effect of the agents.

The exact source of the histamine liberated during anaphylactic shock in rats is not known, but the possible sites of origin have been considerably restricted by the recent experiments of Brocklehurst (1960). In his tests the addition of antigen to the fluid perfusing lung, liver, or hind quarters of sensitized rats did not lead to the appearance of histamine in the perfusate. Since Brocklehurst obtained positive results in similar tests of tissues from other species it may be concluded that the lungs, the liver and hind quarters were not the source of the histamine detected in the blood in our study. The small bowel seems the most likely alternative, particularly since this region shows such a striking vascular change during anaphylaxis. The results of Sanyal & West (1958*a*) support such a possibility. They noted a diminution in the concentration of histamine in the jejunum of rats during anaphylactic shock, although, as they pointed out, this may have been due to dilution owing to oedema.

Maruno (1958*b*) has studied rats given repeated injections of horse serum without an adjuvant. In accord with the variable and minimal reactions associated with subsequent challenge with antigen, he obtained variable and minimal changes in the histamine content of the blood. In some of his animals the histamine and histaminase content of some tissues declined after challenge. It is difficult to correlate these results with those obtained in animals showing the severe reactions produced with the aid of adjuvants.

Rose & Leger (1952) found an increase in the histaminolytic power of the serum of rabbits during anaphylactic shock. Our observations in rats are similar. In neither species has the source of the destroying factor been determined.

In general, the degree of anaphylactic shock, the concentration of histamine, and the histamine-destroying power of the blood were less in rats sensitized after adrenalectomy than in rats sensitized with the aid of *Bordetella* (*haemophilus*) *pertussis* vaccine. The differences may have been due to differences in the degree of sensitization or to differences in amount of histamine or histaminase in the tissues after adrenalectomy (Karady, Rose & Browne, 1940; Rose & Browne, 1941; Valette & Huidobro, 1957; Hicks & West, 1958; Bartlet & Lockett, 1959).

SUMMARY

1. The concentration of histamine in the blood has been estimated under control conditions and during anaphylactic shock in male white rats rendered susceptible to sensitization by the injection of *Bordetella* (*haemophilus*) *pertussis* vaccine and by adrenalectomy.

2. During control studies significant differences in blood histamine were not found between rats anaesthetized with ether and those anaesthetized with pentobarbital sodium; nor were there significant differences in the blood of sensitized and unsensitized animals or of intact and adrenalectomized rats. However, blood drawn by cardiac puncture consistently contained more histamine than blood drawn by venous catheterization.

3. The concentration of histamine in the blood of intact and adrenalectomized animals always increased during anaphylactic shock. During shock free histamine was uniformly detectable in the plasma.

4. During anaphylactic shock in intact and in adrenalectomized rats a histamine-destroying factor, tentatively identified as diamine oxidase (histaminase), was found in the blood.

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INCREASE IN ANTIHAEMOPHILIC GLOBULIN ACTIVITY FOLLOWING INFUSION OF ADRENALINE

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(*Received* 23 *September* 1960)

Christie, Graham-Stewart & Ingram (1960) described an abdomino-perineal excision of the rectum in a haemophilic of moderate severity who received animal antihaemophilic globulin (AHG) to cover the operation and post-operative period. Responses to this material were measured by assaying the AHG activity in the patient's plasma before and after each dose; and on two occasions the responses were strikingly high. These peaks corresponded to episodes of acute haemorrhage requiring immediate blood transfusion; and there was also an intermediate peak coinciding with a lesser haemorrhage insufficient to necessitate transfusion.

It was not clear whether the high responses correlated with the severity of the bleeding or with blood transfusion; but in another haemophilic an unexpectedly high AHG level was associated with haematemesis while the patient was under observation only. It seemed therefore that haemorrhage was the correlated factor; and in considering a possible mechanism, the extensive previous work on the acceleration of blood coagulation after the administration of adrenaline was recalled: this was reviewed by Forwell (1955), and the problem was re-examined by Forwell & Ingram (1957), who thought at that time that the effect depended on an increase in the activity of Factor V.

A possible explanation of the observations in the haemophilics was that haemorrhage had led to a release of adrenaline (Greever & Watts, 1959), and that this had in turn brought about an increase in the activity of AHG in the patients' plasmas. Forwell & Ingram (1957) had not examined AHG in their experimental subjects who had received adrenaline; it therefore seemed of interest to extend the earlier work to include AHG. The results of these experiments are now presented.

METHODS

The healthy experimental volunteers taking part in the present investigation were senior male medical students. The haemophilic subjects were adult patients, also of course, males, and in good health apart from their haemophilia. Neither they nor the healthy subjects were fasted before testing.

The adrenaline, noradrenaline and isopropylnoradrenaline used were the ordinary pharmacopoeial preparations, and were administered by intravenous infusion for periods of 10 min (in some early cases, 15 min) at a rate of $10–16 \times 10^{-6}$ g/min for adrenaline and for noradrenaline (in one instance 20×10^{-6} g/min) and $2–4 \times 10^{-6}$ g/min for isopropylnoradrenaline. Several days elapsed between successive infusions on any one subject.

The general plan of the experiments was to obtain blood samples by venepuncture before and after each infusion, and to compare the post-infusion sample with that taken beforehand. Thus in the AHG assays the post-infusion sample was assayed against the corresponding pre-infusion sample in each case.

During the infusions frequent readings of pulse rate and sometimes of systolic blood pressure were taken by clinical methods.

AHG assays on citrated plasma followed the method of Biggs (1957) using 'undiluted' haemophilic plasma, with the minor modifications given by Christie *et al.* (1960), except that each plasma was tested at three instead of two dilutions (the lowest dilution of test plasma in haemophilic plasma was at least 1 in 10, with step intervals thereafter usually 1 in 3; the actual dilutions depended on the sensitivity of the system on the day of the experiment).

Fibrinogen concentration was determined by a clot-weight method (Ingram, 1952).

Fibrinolytic activity was measured on the lines suggested by Wilhelm, Miles & Mackay (1955), observing serial thrombin clotting times on incubated plasma (Ingram, Norris & Tanner, 1960). A few observations were also made by Bidwell's (1953) method and were generally in agreement.

Blood glucose concentration was determined by Middleton & Griffiths' (1957) method.

RESULTS

AHG responses

Table 1 gives the response of each subject to each drug, as a percentage of the pre-infusion potency. Adrenaline is seen to have produced increases in normal subjects and in mild haemophilics, but not in severe haemophilics. The other drugs produced insignificant effects.

There was no obvious relation between the AHG responses and either the circulatory changes or the subjective effects associated with the three drugs. In both respects adrenaline and isopropylnoradrenaline are rather similar and differ from noradrenaline, yet in their AHG responses noradrenaline and isopropylnoradrenaline were associated and adrenaline was distinct.

It was thought that the AHG response might be associated with the change in blood glucose concentration; but this relationship was complicated by an inverse correlation between the pre-infusion blood glucose concentration and its proportionate increase after infusion: thus, when post-infusion glucose concentration was expressed as a percentage of the pre-infusion value, the correlation coefficients between this percentage and the pre-infusion concentrations were:

for adrenaline, on 12 pairs, $r = -0{\cdot}766$: $P < 0{\cdot}01$;

for noradrenaline, on 6 pairs $r = -0{\cdot}242$: (n.s.);

for isopropylnoradrenaline, on 8 pairs, $r = -0{\cdot}556$: (n.s.);

and the pooled value for all three drugs was $r = -0{\cdot}541$ on 22 pairs: $P < 0{\cdot}01$. Multiple correlation coefficients were therefore calculated for AHG response against glucose response for constant initial glucose concentration for each drug separately and for pooled values, taking the post-infusion glucose concentration both as a percentage of the pre-infusion

TABLE 1. AHG response of each subject to each drug

(i) Normal subjects: responses expressed as percentage potency of pre-infusion samples.

Subject	Adrenaline	Noradrenaline	Isopropylnoradrenaline
WIL	223	94	60
CHE	208	89	92
FEN	208	210	61
LIM	198	106	244
HAR	190	111	140
ING	174	93	175
PEP	155	111	92
HAL	135	99	76
SNA	87	78	106
PHI	225	—	—
PRO	215	—	—
PAW	163	—	—
BAT	135	—	—
Mean	176	110	116

Analysis of variance of the results from the subjects receiving all three drugs did not disclose systematic differences between subjects ($VR = 1{\cdot}06$ on 8, 16, d.f.; $P > 0{\cdot}2$), so that the responses from four additional subjects receiving adrenaline only are included in the mean value for that drug. The mean adrenaline response differs significantly from 100% but the mean responses to the other two drugs do not (S.E. of mean $\times t_8 = 39$, for $P = 0{\cdot}05$).

(ii) Haemophilic subjects: responses expressed as percentage potency of fresh plasma samples pooled from four normal subjects.

Subject	Before adrenaline	After adrenaline
Cal	17	11
Bat	14	40
Arm	12	120
Tin	10	27
But	7·4	15
Fre	4·1	5·3
Wya	0·9	0·9
Sta. S.	0	Trace
Geo. S.	0	0
Mou	0	0

Subject Bat was retested with adrenaline on another occasion, and his AHG activity rose from 13 to 35% of the activity of the pooled normal plasma; however, when tested again with isoprenaline, the pre- and post-infusion values were 13 and 15% respectively. This accords with the findings in normal subjects, *supra*.

value and also in absolute units; none was significant. (The mean initial and final blood glucose concentrations for the groups tested with the three drugs were: adrenaline, 65 and 90 mg/ml.; noradrenaline, 68 and 65 mg/100 ml.; and isopropylnoradrenaline, 57 and 80 mg/100 ml. The

relative effects of the three drugs are in line with previous work: cf. Cobbold, Ginsburg & Paton, 1960. These calculations were made on the data from normal subjects only.)

Possible artifacts

A possible explanation of the AHG response might be that it was spuriously produced by the presence of something in the post-infusion blood samples which accelerated the thromboplastin generation reactions on which the AHG assay is based. Inspection of the assays themselves did not support this idea, because there appeared to be no greater discrepancies in parallelism between the dose–response curves of the pre- and post-infusion samples than between patients' and control curves of clinical assays over the same period of time; thus it would be necessary to postulate that the dose–response curve of the hypothetical accelerator had a slope indistinguishable from that of AHG itself. Nevertheless, this possibility was investigated along two lines.

Direct effects of adrenaline or lactate (see Discussion, p. 223) on the assay system were also investigated *in vitro*, on the lines followed by Forwell & Ingram (1957).

Fibrinolytic activity. It is known that low concentrations of thrombin will accelerate the generation of blood thromboplastin (Biggs & Macfarlane, 1957, Figs. 14, 25). Now thrombin appears to be a proteolytic enzyme (Lorand, 1954), so that critical concentrations of fibrinolysin might have a similar action. Adrenaline administration has been found to promote fibrinolysis (Biggs, Macfarlane & Pilling, 1947; Kwaan, Lo & McFadzean, 1959), and it was therefore possible that, in the present experiments, the AHG response might be no more than an indirect result of fibrinolytic activity. The following attempts were therefore made to show a correlation between these two variables:

1. In six infusions on four subjects (two with each drug) the correlation was tested between the AHG response and the ratio between pre- and post-infusion fibrinogen concentrations in the subject's plasmas: $r = +0{\cdot}57$, implying that both AHG and fibrinogen concentration tended to rise together. Clearly this does not support the hypothesis, for had the AHG response been linked with fibrinogenolysis *in vivo*, the change in fibrinogen concentration should have been negatively correlated with change in AHG activity.

2. In seven infusions in three subjects (two adrenaline, three noradrenaline and two isopropylnoradrenaline) the correlation was tested between the AHG response and the ratio (post-infusion:pre-infusion) of the slopes of the regressions of thrombin time on incubation time in the incubated plasma test: $r = -0{\cdot}19$, implying virtually no correlation; again this does

not suggest any association between the AHG response and fibrinolytic activity.

3. It was thought that such fibrinolytic activity as had been postulated should lead to a change in assay results if the plasmas were incubated for a period before being assayed. In one subject, infused with adrenaline, the pre- and post-infusion plasma samples were immediately divided into two parts. One portion of each was used for AHG assay in the usual way, and the other portions were incubated at 37°C for 30 min and then assayed. A further blood sample was obtained from this subject 2¾ hr after the infusion and treated in the same way. Taking the un-incubated initial sample as 100% activity, the two subsequent unincubated samples gave values of 225 and 175% AHG, while the three incubated samples yielded values of 115, 210 and 187% respectively. The paired results (i.e. those of the corresponding incubated and un-incubated samples) were thought to be in good agreement in each case, so that there was no evidence that incubation had affected the assay value. This result also appeared to be against the proteolytic hypothesis.

A hypothetical substance potentiating AHG. The possibility was also considered that the administration of adrenaline might have led to the liberation of some substance quite unconnected with AHG which might nevertheless have had the property of potentiating the specific activity of AHG in the thromboplastin generation system. Evidence for such a substance was sought in severe haemophilics, whose blood had been shown not to acquire AHG activity after adrenaline infusion (Table 1, ii).

TABLE 2. Adrenaline infusion following the administration of normal plasma to a severe haemophilic (subject Mou)

	AHG activity as % of pooled normal plasma		
Expt.	Immediately after plasma	Before adrenaline	After adrenaline
1	18	23	21
2	32	33	29

The haemophilic subject, Mou., received an infusion of approx. 1 l. of fresh, normal citrated plasma. At the end of the infusion his plasma was assayed against a pool of four normal plasmas; about 2 hr was allowed to elapse and he then received an infusion of adrenaline, his plasma being again assayed, both before and after this infusion, against the same pooled normal plasma. This experiment was carried out on two occasions, and the results (Table 2) show that the adrenaline infusion had little effect on the AHG activity in his plasma.

The haemophilic subject, Geo. S., received an infusion of adrenaline. Dilutions of plasma from an untreated normal subject were then made in

the pre- and post-infusion samples of his plasmas, and the latter assayed against the former. In this way it was thought that it would be possible to detect *in vitro* a potentiation of the normal plasma by the infusion of adrenaline into the haemophilic subject. In fact no potentiation was observed, the second series of dilutions assaying at 107 % against the first.

Effects of adrenaline and lactate in vitro. A specimen of normal citrated blood was divided into four parts and to each was added 1/10th volume of diluent, containing respectively 0, 10^{-5}, 10^{-6} and 10^{-7} g adrenaline per litre. The samples were incubated at 37° C for ½ hr and then assayed for AHG (having been handled up to this point in silicone-coated tubes). Taking the activity of the first sample as 100 %, the subsequent samples yielded potency ratios of 95, 124 and 87 % respectively. This suggested that the transitory presence of adrenaline in the blood, at concentrations of 10^{-6}–10^{-8} g/l., had no detectable effect on a subsequent AHG assay, and was in line with previous findings (Forwell & Ingram, 1957).

Two similar experiments were carried out in which lactic acid was added to normal blood samples so as to cover the range of lactate concentrations from 10 to 40 mg/100 ml.; again, there was no consistent effect.

DISCUSSION

In Forwell & Ingram's (1957) previous work the infusion of adrenaline was found to shorten the whole-blood clotting times in silicone-coated tubes by about 20 % (as compared with 3 % for inert infusions). In seeking to account for this effect the authors demonstrated a moderate increase in Factor V activity (to about 125 % of pre-infusion levels). The present evidence of an increase in AHG activity (to about 175 % of the pre-infusion level) seems to offer a more reasonable explanation for the shortening of the clotting time, but perhaps both effects contribute. It appears that AHG enters the clotting sequence at an earlier stage than Factor V (Bergsagel & Hougie, 1956), and this is an additional reason for thinking that an increase in AHG activity accounts more satisfactorily for the hypercoagulability produced by adrenaline; for the earlier stages of clotting are more time-consuming than the later (Biggs, 1955), and thus, if accelerated, offer a greater scope for shortening the whole clotting time.

On the other hand the present work by no means excludes the possibility that the AHG response is artifactual. A good test for the reality of the increase in AHG activity would be to show that plasma from adrenaline-treated donors was more effective in arresting haemophilic bleeding than an equal quantity from untreated donors. This test would, however, involve the clinical assessment of materials whose relative potency would not be expected to differ by more than a factor of 2.

It is interesting that there appear to be appreciable differences in the

normal AHG levels among different races of man, and that in tested groups of both Bantu (Merskey, Gordon & Lackner, 1960) and Australian aborigines (Pitney & Elliott, 1960), the mean AHG level was very close to the mean increase here reported in Europeans after adrenaline infusions. Pitney & Elliott (1960) also found a similar mean level in 22 white Australians with hyperglobulinaemia. The relationship between these findings is obscure, but it would perhaps be worth assaying plasma AHG under a wider range of circumstances.

The pattern of the AHG response to the three drugs here tested is similar to their effect on blood lactate, which rises after adrenaline (Barcroft & Cobbold, 1956) but not after noradrenaline or isopropylnoradrenaline (Bearn, Billing & Sherlock, 1957; Cobbold *et al.* 1960).

SUMMARY

1. The intravenous infusion of adrenaline raised the potency of antihaemophilic globulin in the plasma of normal subjects and of mildly, but not severely, affected haemophilics. Noradrenaline and isopropylnoradrenaline did not have this effect.

2. The occurrence of this effect was not associated with the clinically observable circulatory effects of the three drugs, nor with their effect on blood glucose concentration, but it followed the same pattern as their known effect on blood lactate.

3. Attempts to explain the rise in AHG activity on the basis of artifacts produced by fibrinolytic activity or a non-AHG potentiator were unsuccessful.

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THE SEPARATION OF THE SMOOTH-MUSCLE STIMULANTS IN MENSTRUAL FLUID

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A group of lipoid stimulants of smooth muscle is found in human menstrual fluid. They are thought to be formed in the endometrium and to be released during menstruation, when they pass into the menstrual fluid, into the circulating blood and perhaps also by diffusion to the adjacent layers of myometrium. By either or both of the latter routes they are thought to stimulate the menstrual contractions of the myometrium (Pickles, 1957, 1959*b*). Their presence in the circulating blood during menstruation (Pickles, 1959*a*) may also affect smooth muscle elsewhere, for example in the gastro-intestinal tract (McCance & Pickles, 1960).

In a previous account (Chambers & Pickles, 1958) some evidence was given that the menstrual fluid contained at least two stimulant lipids, which could be partially separated by chromatography. We now know that the extracts contain at least three such substances, which can be clearly separated from each other. Until they are identified chemically these are referred to as components *A*, *B* and *C*, in decreasing order of polarity. In addition, some extracts contain a fourth stimulant; but this is an artifact, and has been shown to be formed by the hydrolysis of a constituent of the absorbent material on which the menstrual fluid is collected. This paper describes the methods of separating these stimulants from one another and from the non-stimulant substances in the extracts.

METHODS

Menstrual fluid extracts. Most of the subjects were students at a residential college and were known to be in normal health. About 1200 'specimens' of menstrual dressings were collected in polythene vessels each containing 30 ml. of A.R. acetone and bearing a label on which the donor recorded particulars of date, time and also the type of absorbent material used. The latter were of several kinds, and included some which were specially prepared by the manufacturers free from the compound from which the 'artifact', ricinoleic acid, originated (p. 230). Menstrual fluid was also collected by one subject on absorbent material which had been previously washed with water, acetone, ether and petroleum ether.

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The specimens were brought to the laboratory daily and extracted with 3 × 10 ml. acetone each, in batches of 6–20, either immediately or after storage for not more than a few days at −20° C. After evaporation of the acetone under reduced pressure in a rotary evaporator, the lipids were dissolved in diethyl ether of anaesthetic grade, or more often in dichloromethane, in order to include all of the most polar component, *A*. The solution was filtered and the solvent evaporated off, leaving a residue referred to as the 'crude lipid extract'.

Several extracts were similarly made of secretory endometrial curettings, which were taken directly from the uteri into dry glass containers, brought immediately to −20° C, and kept at that temperature until extracted. All extracts were stored at −20° C.

It had previously been found (Pickles, 1959*b*) that no appreciable quantities of histamine, acetylcholine or 5-hydroxytryptamine, added to the initial acetone extract, passed into the crude lipid extract (then made with ether). A similar experiment showed that this was still true for histamine and 5-hydroxytryptamine when dichloromethane was used instead of ether, but that a small amount of acetylcholine could pass into the crude extract. In a further experiment 10 mg of acetylcholine was put along with 120 mg of crude lipid extract on a silica column, and fractionally eluted as described below. Tests with a sensitive non-atropinized guinea-pig terminal ileum preparation failed to show the presence of acetylcholine in any of the fractions eluted from the column with lipid solvents; but acetylcholine was recovered later in an additional fraction eluted by means of water. Although it was probably an unnecessary precaution, $2–5 \times 10^{-8}$ g of atropine, mepyramine and bromolysergic acid per millilitre were nevertheless added to the bath solution in most of the tests on uterine muscle described below.

Fractionation of the extracts

Partition between heptane and aqueous methanol. The crude lipid extracts were dissolved in heptane, 1 ml./10 mg extract, and shaken successively with 3 × 1 vol. of 80% methanol in water. The residues after evaporation of the solvents are called the 'heptane' and 'aqueous-methanol' fractions. This method was commonly used in the processing of large batches (> 250 mg) of crude extract, in order to avoid overloading the silica-gel column during the separation of components *A* and *B* (below).

Fractional elution from silica gel. Columns were prepared by packing a slurry of dry silica gel (grade 'for chromatographic absorption', British Drug Houses, Ltd) in petroleum ether (b.p. 60–80° C), into a tube 2·0 cm diameter to a height of 10 cm. Crude lipid extracts or aqueous-methanol fractions were put on the columns and eluted successively with 200 ml. each of the following solvents: *a*, petroleum ether (b.p. 60–80° C); *b*, 30% diethyl ether in petroleum ether; *c*, diethyl ether; *d*, 7½% methanol in ether; *e*, 30% methanol in ether; *f*, 60% methanol in ether. The principal chromatographic fractions are always referred to in this paper by the above letters. Since aqueous-methanol fractions were largely insoluble in petroleum ether, they were commonly applied in solvent *b* and elution was begun with this solvent. Amounts of lipid in excess of 250 mg overloaded the column and sometimes gave anomalous results. For chromatography of less than 25 mg of lipid, smaller columns 1·3 cm diameter and 10 cm height were used, eluted with 100 ml. of each solvent mixture. Such columns were particularly used for the further chromatography of acidic lipids prepared as described below.

Extraction of acidic lipids by $NaHCO_3$. This method was particularly useful for chromatographic fractions *b* and *c* obtained as described in the preceding section. The residues of the eluted lipids of fraction *b* were dissolved in heptane or in ether (1 ml./10 mg lipid) and shaken successively with 3 × 1 vol. of $NaHCO_3$ solution 2·6 g/100 ml. The combined aqueous phases were filtered and brought to about pH 4 with H_2SO_4, and shaken successively with 2 × 1 vol. of the organic solvent, which was then evaporated off leaving a residue of acidic lipids. In experiments in which the chromatographic fraction *b* had been prepared from crude lipid extract not submitted to heptane-methanol partition, the 'non-acidic' residue left in the

heptane or ether after shaking with $NaHCO_3$ was used as one source of component *C* (see 'Chromatography on partially hydrated alumina', below). As the acids in fraction *e* were sparingly soluble in ether, dichloromethane was used instead of ether in processing this fraction.

Partition chromatography on kieselguhr. Indicator columns of 'Hyflo Supercel' kieselguhr (Johns, Manville and Co.) were prepared as follows: Petroleum ether 250 ml. was shaken with methanol 85 ml. and water 15 ml. and the layers were allowed to separate. Bromocresol green 3 mg was dissolved in 20 ml. of the lower (aqueous methanolic) phase; 1 drop of conc. ammonia solution was added to another 20 ml. of this phase; the latter solution was then added drop by drop to the former until the indicator just turned green-blue. To 20 g Hyflo Supercel suspended in 200 ml. of the upper phase was added 6–8 ml. of the indicator solution, and the mixture was shaken vigorously until free from lumps. The mixture was run into the column and the kieselguhr was packed by air pressure from above. The lipids were added and eluted by petroleum ether or mixtures of it with diethyl ether.

Chromatography on partially hydrated alumina. Alumina (Woelm neutral, activity 1) was hydrated by adding 10% of water, and after 24 hr or more in a well-stoppered vessel, to allow the water to become evenly distributed, the alumina was packed in A.R. benzene into columns 10 cm × 1·3 cm diameter. The lipids of heptane fractions prepared by heptane-methanol partition, or the non-acidic residues of chromatographic fraction *b* prepared from crude lipid extracts, were applied in benzene. They were eluted fractionally by 20 ml. lots of the solvent mixtures 0, 10, 20...90, 100% diethyl ether in benzene.

Chromatography on cellulose powder. Whatman Cellulose Powder was suspended in acetone-ether mixtures and allowed to settle without forcible packing, to give columns 7 cm in height and 1·3 cm in diameter. Acidic lipids from chromatographic fraction *e* were added in a small volume and eluted with 5 ml. lots of the same solvent mixture.

Paper chromatography. Ascending chromatograms were run for 2–4 hr in the upper phase of the mixture ethyl methyl ketone–water–diethylamine in the proportion 60–20–3 (Vogt, 1955; Ambache, 1959). For biological testing, the stimulants were extracted from strips of chromatograms by means of ether that had been shaken with dilute HCl.

'Blank' and other control extracts. The following control extracts were prepared. (1) Unused absorbent dressings of all the main kinds used by the subjects were moistened with water or NaCl solution 0·9 g/100 ml. and then put through the usual extraction procedure; these are called 'blank' extracts. (2) In two instances the dressings were incubated with venous blood at 37° C for several hours before the lipids were extracted. In one of these experiments the venous blood was taken on the second day of the donor's menstrual period.

Smooth-muscle preparations

Uterine horns from guinea-pigs of 350–500 g were suspended at 37° C in a 10 ml. bath containing an oxygenated solution of the following composition (g/100 ml.): NaCl 0·82, KCl 0·052, $CaCl_2$ 0·008, $NaHCO_3$ 0·047, $MgSO_4$ 0·018 and glucose 0·1. The amount of $MgSO_4$ was sometimes doubled or halved in order to give a convenient amount of spontaneous rhythmical activity in the preparation, since the effect of the menstrual stimulants was most prominent when the spontaneous contractions were small but not absent. In some of the tests guinea-pig duodena or human myometrial strips were suspended in the same solution. Guinea-pig seminal vesicles, periodically stimulated with noradrenaline, were used to test for the factors responsible for the potentiating effect, of the type shown by the 'chromatographic fraction 4' described by Chambers & Pickles (1958).

Formal bioassay methods could not be used, since the available quantities of stimulants were usually too small for the tests to be repeated many times; moreover the extracts under comparison usually contained different substances, rather than different quantities of the same stimulant. The term 'activity' is therefore used here in a general sense, the activity of an extract being measured inversely by the concentration of it that caused a moderate

contraction of a guinea-pig uterine horn on application for 2–3 min. In order to avoid the appearance of greater accuracy than the tests justified, the effective concentrations (*E.C.*) producing such a response have been selected, in giving the results, from the following series: 1, 3, 10, 30, 100, 200 μg/ml. If the uterus did not respond to the highest concentration of a particular extract that was applied, the *E.C.* is recorded as this concentration preceded by the symbol '$>$'. The *E.C.* of (+)-ricinoleic acid was usually 3–10 μg/ml.

RESULTS

Quantities of crude lipid extracted on successive days of the menstrual period. The amount of ether-soluble crude lipid extracted from the menstrual dressings varied between 3 and 11 mg/hr of flow, with a mean of 6. In two experiments the initial acetone residue was re-extracted first with ether and then with dichloromethane, which extracted a further 0·3–0·4 mg of 'crude lipid' per milligram of ether-soluble material.

In one experiment a series of 23 specimens, from a restricted number of subjects, was extracted in three batches according to the day of the period. The mean amount of ether-processed crude extract was 5·7 mg/hr for the first day and 6·3 mg/hr for the second, but only 3·4 mg/hr for the third. The activity of the third-day extract was also less than that of the others. A further complete series of specimens from one subject showed a gradual decrease in total activity from the first day to the fourth. One half the total number of specimens in the main series were collected on the first day of the period, and only 15 % on the third or later days.

Concentration of the activity by heptane-methanol partition. About $\frac{1}{3}$–$\frac{1}{6}$ by weight of the total crude lipid extracts passed into the aqueous methanol, but the activity of this fraction was 3–10 times that of the heptane fraction. Heptane-methanol partition was therefore commonly used as a first step in concentrating the more polar stimulants, components *A* and *B*; and the less polar component *C* could be recovered from the heptane fraction. Further partition of the methanol fraction between 80 % aqueous methanol and heptane-benzene (60:40) mixture gave some further concentration of components *A* and *B* but some activity was lost into the less polar phase, and this method was not routinely used in the preparation of stimulant concentrates.

On countercurrent distribution of crude extracts between heptane (stationary phase) and 80–92 % aqueous methanol, the activity was distributed over a wide range of the methanol fractions, suggesting that at least two stimulants were present but not clearly separated.

Fractional elution from silica gel. Typical results obtained by this method are shown in Table 1. Column (i) shows the fractionation of 250 mg of dichloromethane-soluble crude lipid extracted from 19 menstrual specimens; and, for comparison, column (ii) shows the fractionation of a 'blank'

control extract consisting of 110 mg of lipid extracted by the same method from 20 unused dressings of the same kind as in (i). Column (iii) also for comparison with (i) shows the result of first eliminating much non-polar inactive material (together with some component *C*) from a large batch of crude lipid extract that would otherwise have overloaded the silica-gel column. This extract consisted of 700 mg of crude lipid from 40 menstrual dressings of the same kind. On partition it yielded 130 mg of aqueous-methanol fraction, which was chromatographed as shown.

In all instances chromatographic fraction *a* was inactive and was not examined further. Its amount was greatly reduced in the aqueous-methanol fraction (Table 1, iii).

TABLE 1. Examples of fractional elution from silica-gel columns: for details see text

Fraction	Solvent mixture	Principal stimulants	(i) Menstrual crude extract (250 mg from 19 specimens)		(ii) Blank crude extract (110 mg from 20 specimens)		(iii) Menstrual aqueous-methanol fraction (130 mg from 40 specimens)	
			(mg)†	*E.C.** (μg/ml.)	(mg)†	*E.C.** (μg/ml.)	(mg)†	*E.C.** (μg/ml.)
a	Petroleum ether (b.p. 60–80° C)	—	3·63	> 100	0·77	> 200	0·31	> 100
b	30 % diethyl ether in petroleum ether	*B, C*	6·23	100	2·29	200	0·75	30
c	Diethyl ether	Artifact	1·01	30	1·10	30	0·59	10
d	7½ % methanol in ether	(Unidentified)	0·67	100	0·55	100	0·22	30
e	30 % methanol in ether	*A*	1·31	30	0·61	> 100	0·89	30
f	60 % methanol in ether	(Unidentified)	0·34	100	0·18	> 100	0·44	> 100

* Effective concentration on guinea-pig uterus. † Wt. of each fraction *per specimen*, for comparison of menstrual extract with blank.

Fraction *b* always contained some activity, even in the blank extracts; but the amount of activity in the whole fraction was greater in the menstrual than in the blank extracts. Thus in the examples shown in Table 1, this fraction contained 3–6 effective doses per specimen (tested on the guinea-pig uterus *in vitro*) in the experiments of columns (i) and (iii), but only 1 dose in the case of the 'blank', column (ii). This difference became more marked on re-chromatographing the fraction *b* lipids with a different series of solvents, as in the example shown in Table 2. Here it is apparent that both the menstrual and the blank extracts contained a stimulant eluted by 20–30 % diethyl ether in petroleum ether, but the menstrual extract contained a less polar stimulant eluted by 10–15 % ether (fractions *b*2 and *b*3). In the experiment of Table 2, the crude lipid had not been

first partitioned between heptane and aqueous methanol; but other experiments showed that the heptane and the aqueous-methanol fractions of menstrual dressings both contained stimulants that could be eluted from silica-gel by 10–15 % ether. These stimulants differed in some other properties, as described below; the one found mainly in the heptane fraction is referred to as component *C*, and the one found mainly in the aqueous-methanol fraction as component *B*.

TABLE 2. Further concentration of components *B* and *C* by re-chromatography on silica gel

Fraction	Solvent mixture (%)*	Menstrual extract (mg)†	Menstrual extract *E.C.*‡ (μg/ml.)	Blank extract (mg)†	Blank extract *E.C.*‡ (μg/ml.)
*b*1	5	0·96	> 100	0·38	> 100
*b*2	10	1·67	30	0·49	> 100
*b*3	15	0·64	30	0·33	> 100
*b*4	20	0·16	100	0·13	30
*b*5	25	0·12	30	0·19	30
*b*6	30	0·18	30	0·17	30

Crude menstrual lipid 490 mg, extracted from 21 specimens, was first chromatographed as in Table 1. Fraction *b*, 83 mg, was re-chromatographed on silica gel. The blank extract was made similarly from 20 unused specimens of the same kind.

* Diethyl ether, %, in petroleum ether, b.p. 60–80° C. † Wt. of each fraction *per specimen*, for comparison of menstrual extract with blank. ‡ Effective concentration on guinea-pig uterus.

Chromatographic fraction *c* showed no significant difference, either in weight or in activity, between the menstrual and the 'blank' extracts. The principal stimulant in it has been identified as (±)-ricinoleic acid (R. F. C. Brown, unpublished), and is a hydrolysis product of a constituent of the unused dressings. It is therefore an artifact of no physiological interest. The small amount of activity in chromatographic fraction *b* of unused dressings is attributable to the same or a very similar artifact. Blank extracts of unused dressings that had been specially manufactured without the addition of the constituent from which this artifact arose still contained some stimulant substance in fraction *c*; but these extracts were of much smaller activity than the normal blank extracts. The substance causing this residual activity has not been identified.

Chromatographic fraction *d* of menstrual extracts was usually smaller in both amount and activity than fractions *c* and *e*, and in such cases it was not examined further. In some experiments, however, especially when large quantities (> 200 mg) of crude lipid had been put on the column, fraction *d* contained more total activity than fraction *e*. In such cases fraction *d* was re-chromatographed, and the activity now appeared more in fraction *e*. One batch of silica-gel also gave component *A* in fraction *d* even

when the column was not overloaded. All the blank extracts showed only a trace of activity in fraction *d*.

Fraction *e* contained the principal and most polar menstrual stimulant or stimulants, referred to as component *A*. The corresponding fractions of blank extracts contained at most a very small trace of activity.

Fraction *f* was always of low activity and was not examined further.

Further concentration of component A. From each 1 mg of fraction *e* lipids, approx. 0·14 mg of acid substances could be extracted by means of $NaHCO_3$ as described under 'Methods'. These acids were of much higher stimulant activity than the non-acid residues. The activity could be still further concentrated by re-chromatography on silica gel, from which the

TABLE 3. Re-chromatography of component *A* on silica gel

Fraction	Solvent mixture methanol % in diethyl ether	Menstrual extract (mg)*	Menstrual extract *E.C.*† (μg/ml.)	Blank extract (mg)*	Blank extract *E.C.*† (μg/ml.)
*e*1	0 (= ether)	0·026	> 10	0·011	30
*e*2	10	0·032	3	0·011	> 30
*e*3	20	0·019	3	0·009	> 30
*e*4	30	0·013	3	0·010	> 30
*e*5	40	0·015	10	0·005	> 30

Crude menstrual lipid 1, 850 mg, extracted from 52 specimens, yielded 286 mg of aqueous-methanol fraction, from which 71 mg of chromatographic fraction *e* was prepared. From this, 8·2 mg of acidic lipids were extracted, which were re-chromatographed as above. The blank extract was made similarly from 30 unused specimens of the same kind.

* Wt. of each fraction *per specimen*, for comparison of menstrual extract with blank.

† Effective concentration, in μg/ml., on guinea-pig uterus.

activity was eluted by 10–30 % methanol in ether, as in the example of Table 3. Component *A* could also be further concentrated by chromatography on cellulose-powder columns eluted with acetone-ether mixtures. The activity always appeared in one group of adjacent tubes, as in the example of Fig. 1.

Further concentration of component B and its separation from the artifact. The acids extracted from chromatographic fraction *b* of either menstrual crude lipid or its aqueous-methanol fraction were always more active than the non-acid residues. The fraction *b* acids of blank extracts also contained a little stimulant activity, but much less than in the menstrual extract. When the acids of menstrual fraction *b* were chromatographed on kieselguhr containing an indicator, development with petroleum ether clearly separated two acids: one, component *B*, was eluted by the first 10 ml. of the solvent, and proved to be a potent stimulant of the guinea-pig uterus; the other moved more slowly, and was possibly identical with the artifact (±)-ricinoleic acid found mainly in fraction *c* and to a smaller extent in the

fraction *b* acids of blank extracts. If the amount of acid was too small, it was not possible to detect any colour change in the indicator on the column, but the first 10 ml. of eluate always contained component *B*.

Further concentration of component C. This was found mainly in the heptane fractions of menstrual extracts; the heptane fractions of blank extracts had little or no detectable activity. It was concentrated by

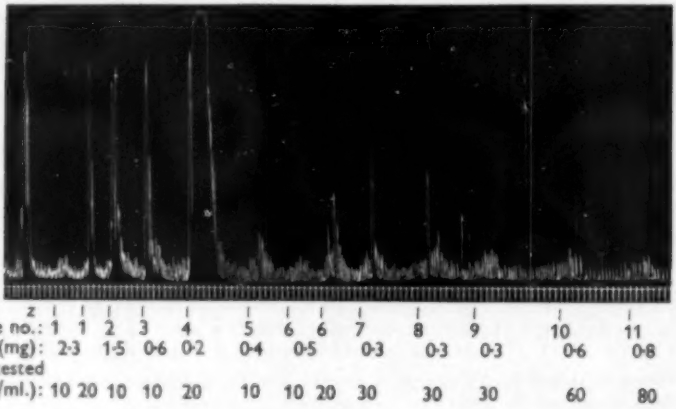

Fig. 1. Chromatography of component *A* on cellulose powder. Approx. 8 mg of acidic lipids of silica gel chromatographic fraction *e* were put on a cellulose powder column 10 cm × 1·3 cm in 20 % acetone–80 % diethyl ether and eluted with the same solvent, 5 ml. to each tube. Total wt. of each fraction as shown above. At *z*, 20 μg/ml. of another extract containing component *A* to test sensitivity of the guinea-pig uterus preparation. Stimulants washed out as soon as preparation showed a definite response, or after 4 min if no response. Most of the activity appeared in tubes 2–4, which comprise 30 % of the total weight of lipid. Time marker, minutes.

chromatography on partially hydrated alumina. Elution with 10–40 % ether in benzene first removed much relatively inactive material, after which 50–90 % ether in benzene removed a small amount of the active component *C*. An example is given in Table 4.

Paper chromatography of component A. In the system methyl ethyl ketone–diethylamine–water (60:–3:–20 by vol.) described by Vogt (1957), component *A* ran to a region which varied in different experiments between R_F 0·3 and 0·9, whereas ricinoleic acid ran to R_F 0·8–1·0. After prolonged development, even in nitrogen, recovery of activity was often poor. Chromatograms of some of the less highly purified extracts showed also fluorescent or coloured spots of lower R_F value than component *A*, but these were never active on elution. Paper chromatography was used in

supplementary tests of the purity of preparations of component *A*, but it was not used for preparative purposes.

TABLE 4. Fractional elution of component *C* from alumina column

Solvent mixture (% ether in benzene)	Weight of each fraction (mg)	Effective concentration
0 (= benzene)	1·7	*
10	8·8	*
20	59·6	*
30	4·2	*
40	1·3	*
50	0·7	†
60	1·1	†
70	1·3	100
80	0·8	30–100
90	1·0	100
100 (= ether)	1·4	†

Heptane fraction 902 mg was chromatographed on silica, and 128 mg eluted by 10–15% ether in petroleum ether contained some stimulant. After extraction of the acids with $NaHCO_3$, 101 mg of the 'non-acid' was chromatographed on alumina, eluted with 20 ml. of each successive solvent. The corresponding 'blank' extract fractions were all inactive.

* These fractions (100 μg/ml.) had a small effect, but only after being washed out of the organ bath. † These fractions caused only small responses when the whole of each was put into the 10 ml. organ bath.

Blank and other control extracts

All the eight kinds of unused absorbent material that were tested contained lipid material extractable and fractionated by the methods described. The quantities of blank crude lipid extracts were commonly $\frac{1}{4}$–$\frac{1}{3}$ of the quantities in the corresponding menstrual extracts, when equal dry weights (obtained by weighing the dried absorbent materials after the lipids had been extracted) of used and unused absorbent materials were compared; but in some fractions, e.g. Table 1, fraction *c*, the 'blank' component appeared to account for the whole of the menstrual fraction. Therefore, only differences between a menstrual fraction and the corresponding blank fraction have been taken as significant.

The differences in activity were greater than the differences in weight. The activities of the crude blank extracts varied from an undetectable amount to $\frac{1}{8}$ of that of the corresponding crude menstrual extract; and there were additional differences in dose–response curves between the menstrual and the blank extracts. Although traces of activity were detected in all chromatographic fractions *b*–*e* of a blank extract when very high concentrations ($\frac{1}{2}$–2 mg/ml.) were tested, almost all the activity of the blank extracts was found in fraction *c*. Most of this activity could be accounted for by the known content of (±)-ricinoleic acid; but small amounts of other stimulants may also have been present in the blank extracts. Extracts of menstrual fluid collected on washed material, and

extracts of fresh secretory endometrial curettings which must have been free from artifacts of this kind, still contained some stimulant in chromatographic fraction *c* as well as in *b*, *d*, and *e*; but the amount was not enough to enable it to be examined further. The use of the special 'artifact-free' absorbent material (p. 226) facilitated the extraction of the fraction *b* and fraction *e* stimulants.

The samples of unused absorbent material that were incubated with venous blood (in one instance taken from a menstruating subject) gave about twice as much crude lipid extract as the corresponding simple blank extracts; but this was nevertheless only about half the amount obtained from used material of the same kinds, and the distribution and potency of the stimulant in it did not appreciably differ from the simple 'blank' extracts. This shows that the stimulants in the menstrual extracts did not originate from some interaction between blood and the absorbent materials.

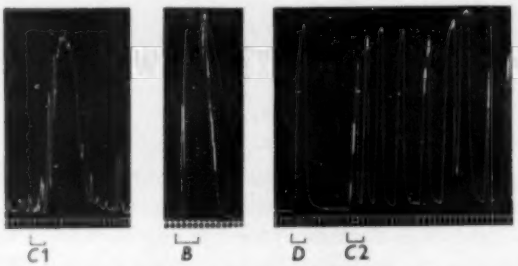

Fig. 2. Responses of guinea-pig uterine horns to components *B* and *C*. At *C*1 33 μg/ml. and at *C*2 100 μg/ml. of extracts containing component *C* applied for 3 min; at *B* 50 μg/ml. of a component *B* preparation applied for 4 min. Responses to component *C* are slower than to component *B*; the response to *C*2 persisted more than 1½ hr after washing out, although, as at *D*, the tissue had given transient responses to an extract containing mainly component *B*. Time marker, minutes.

The amount of stimulant activity found in the venous blood of a menstruating subject (Pickles, 1959*a*) was also much too small to account for the activity of the menstrual extracts on the assumption that this originated from the blood forming part of the menstrual fluid.

The extracts of endometrial curettings contained substances with the properties of components *A*, *B* and *C* (see also Clitheroe, 1961).

Biological effects of these stimulant fractions. The general types of effect on smooth-muscle preparations have been described before (Chambers & Pickles, 1958; Pickles, 1959*b*) and only the following additional facts need be noted here. Although all these stimulants caused contractions that were slow and persistent by comparison with those induced by histamine, the responses to component *C* were often delayed in onset by ½–2 min and then

persisted for 5 min or more, even after the stimulant had been washed out (Fig. 2). The most potent preparations that were made of components *A* and *B* had at least twice the stimulant potency per unit weight of (+)-ricinoleic acid. Component *C* preparations were less active. Crude lipid extracts potentiated the effect of noradrenaline on the guinea-pig seminal vesicle. This effect was given by components *A* and *B* but not by *C*, and it resembles that of prostaglandin (Goldblatt, 1935).

DISCUSSION

The menstrual stimulants, defined as the group of lipoid smooth-muscle stimulants in menstrual fluid (Pickles, 1959*b*), comprise at least three clearly separable substances. They are called 'components' since it is possible that each may later prove to consist of more than one stimulant substance, which have not been separated by the methods described here.

Component A passes into aqueous methanol on partition with heptane. It can be extracted from organic solvents by aqueous $NaHCO_3$ solution, and back into an organic solvent from the aqueous phase after acidification; it is therefore acidic. It is eluted from silica gel by 10–30% methanol in ether but not readily by less polar solvents, and it is very readily eluted from a cellulose-powder column by 20% acetone in ether.

Component B also passes into aqueous methanol on partition with heptane, and like component *A* it behaves as an acid on partition between organic and aqueous phases of different pH values. It differs from component *A* in that it can be eluted from silica gel by 10–15% diethyl ether in petroleum ether, and it is readily removed from a kieselguhr column by means of petroleum ether.

Component C passes mainly into heptane on partition between heptane and aqueous methanol. It does not appear to be acidic, since it cannot be extracted from organic solvents by means of aqueous $NaHCO_3$ solution, but it can be eluted from alumina by ether-benzene mixtures.

Many extracts also contain a stimulant which is an artifact. This is intermediate in its solubility in polar solvents between components *A* and *B*, and it has been identified as (±)-ricinoleic acid. It originates in a constituent of the absorbent materials on which the menstrual fluid is necessarily collected, accounts for only a small part of the total stimulant potency of the menstrual extracts, and is of interest only in so far as it may be confused with the menstrual stimulant components.

Although the methods described here do not necessarily provide us with these components in a pure state, or entirely free from small amounts of other stimulants, the principal components can be separated from each other and from non-stimulant substances well enough for some further chemical analysis to be done.

It is likely that the stimulants which Chambers & Pickles (1958) sometimes found in their chromatographic 'Fraction 3' were in fact those described here as components *B* and *C*. Their 'Fraction 4' is likely to have contained component *B* and the artifact (±)-ricinoleic acid. The main stimulant in their 'Fraction 5' was almost certainly component *A*.

SUMMARY

1. Three principal components of the menstrual stimulant group have been separated by the following methods, which are described in detail.

(*a*) On partition of crude lipid extracts between heptane and aqueous methanol, most of the activity passed into the methanol.

(*b*) Fractional elution of the lipids from a silica-gel column partially separated the stimulants from one another and from much non-stimulant material.

(*c*) Most of the stimulant behaved as acid on partition between organic and aqueous phases of different pH values, but one component, in a relatively non-polar silica-gel fraction, did not.

(*d*) This non-acidic component, *C*, could be separated from some non-stimulant material by fractional elution from partially hydrated alumina.

(*e*) The less polar acidic component, *B*, could be further purified by chromatography on kieselguhr.

(*f*) The more polar acidic component, *A*, could be further purified by chromatography on cellulose powder.

(*g*) Paper chromatography was used in supplementary tests of purity.

2. A further stimulant was found in some extracts, and was identified as an artifact.

3. The quantities of these stimulants and their relative biological effectiveness are described.

We wish to record our thanks to Professor R. A. McCance for his interest; to the Medical Research Council for a personal grant to V.R.P.; to Miss A. Smart, Mrs J. McDermott, Miss P. Hennessy and others for invaluable help in the collection of the specimens; to Southalls (Birmingham) Ltd for supplying the special absorbent material; to Smith and Nephew Research Ltd for a grant for technical assistance; and especially to the many donors of biological material.

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OBSERVATIONS ON THE MECHANISM OF THE REDUCTION IN DISTENSIBILITY OF THE LOW-PRESSURE VESSELS OF THE HUMAN FOREARM DURING OVERVENTILATION

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Factors which alter the distensibility of blood vessels are of importance, since the maintenance of the circulation depends upon the adjustment of the total capacity of the blood vessels to the volume of circulating blood. It has been shown by Duggan, Love & Lyons (1953), Page, Hickam, Sieker, McIntosh & Pryor (1955), and Burch & Murtadha (1956) that segments of superficial arm veins, isolated by compression so that internal pressure may be measured at constant volume, are very reactive to such stimuli as a deep inspiration, the Valsalva manoeuvre, and body cooling and tilting. On the other hand, plethysmographic measurements on entire limb segments (Greenfield & Patterson, 1956; Litter & Wood, 1954) indicate that the distensibility of the low-pressure capacity vessels is sometimes little changed by stimuli which produce large alterations in blood flow (Blair, Glover & Kidd, 1959; Kidd & Lyons, 1958).

Knowledge of the central control of venous distensibility is very incomplete. Using a plethysmographic method with venous congestion Eckstein, Hamilton & McCammond (1958) have demonstrated a constriction of the capacity vessels of the forearm during overventilation with air or 5% carbon dioxide. It seems probable that this constriction depends upon the stimulation of a hypothetical central receptor or group of receptors. The present experiments were designed to verify the findings of Eckstein, Hamilton & McCammond, and to identify, if possible, the particular disturbance associated with overventilation which is responsible for the changes in behaviour of the capacity vessels. It was hoped that this might give an indication of the nature of the effective central stimulus.

METHODS

Experiments were carried out on sixteen healthy male students whose ages ranged from eighteen to twenty-three years. Each subject lay supine on a couch with the forearm slightly raised so that its axis was in the same horizontal plane as the sternal angle. The forearm was enclosed in a water-filled plethysmograph, of the type described by Greenfield (1954), maintained at 35° C. A pneumatic cuff around the wrist was inflated to 200 mm Hg; a second

cuff on the upper arm was adjusted carefully so that when inflated to 50 mm Hg no initial displacement artifact was seen. Volume changes were recorded by a float chamber writing upon smoked kymograph paper. The manometer scale of the proximal cuff was adjusted so that the zero mark was at the pressure at which venous filling became just measurable when the cuff was gradually inflated. This 'titrated zero', level (Litter & Wood, 1954) was used throughout the experiment. A first recording was made after the subject had been lying at rest for 20 min. The proximal cuff was rapidly inflated to a reading of 50 mm Hg above the titrated zero and maintained there until the rate of volume increase became very small. The pressure was quickly reduced to 40 mm Hg for 15 sec and then down to zero by 5 mm stages, each lasting 15 sec. A typical record is shown in Fig. 1.

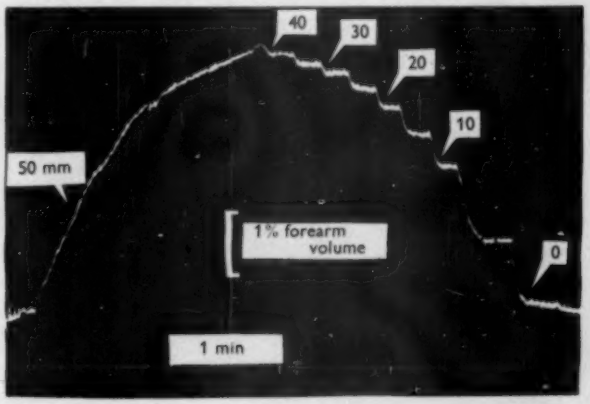

Fig. 1. Typical record of forearm volume changes. The numbers represent the congesting cuff pressures in mm Hg.

Reliability of method. In addition to the usual difficulties in plethysmography, such as stability of the limb, avoidance of leaks and air bubbles, and maintenance of constant temperature, a possible source of error in the method might be that the record obtained at each pressure level is of insufficient duration to provide a reliable measurement of volume change. It was found, however, that when the cuff pressure was maintained any step level could be prolonged with little variation of volume for an additional 4 min. It is also possible that this method gives results different from those obtained by the more usual method of inflating the proximal cuff from zero and measuring the volume increase about 4 min after the application of each pressure. Both methods were used in two experiments on a resting forearm, and the results are shown graphically in Fig. 2. Similar results were obtained by the two methods, but the 'step-down' method has the advantage that the measurements occupy less time.

First series of experiments

The experiment on each subject consisted of a record taken during overventilation of air followed by two records taken during normal breathing; the wrist cuff was then deflated for 2 min. The experiment was repeated with overventilation of 4 % CO_2, and then 9 % CO_2.

Overventilation with air. The subject had previously accustomed himself to breathing at 30 l./min. He was instructed to breathe at this rate through a mouth-piece and valves. The overventilation lasted about 3 min., commencing when venous filling was nearly complete at a cuff pressure of 50 mm Hg, and ceasing 15 sec after return to zero pressure.

Expired air was collected in a Douglas bag during the period of overventilation, in order to determine the degree of overventilation.

Overventilation with 4 % *carbon dioxide.* The subject was instructed to overventilate from a 100 l. Douglas bag containing 4 % carbon dioxide in air, at the same rate as before and using the same taps and connecting air-pipes.

Administration of 9 % *carbon dioxide.* Without warning the subject, a Douglas bag containing 9 % carbon dioxide in air was connected to the mouth-piece; the subject was not instructed to overventilate voluntarily. It was found that respiration gradually increased to a rate of about 30 l./min; graded decreases in cuff pressure were then commenced, and the tap opened to collect expired air in a second Douglas bag.

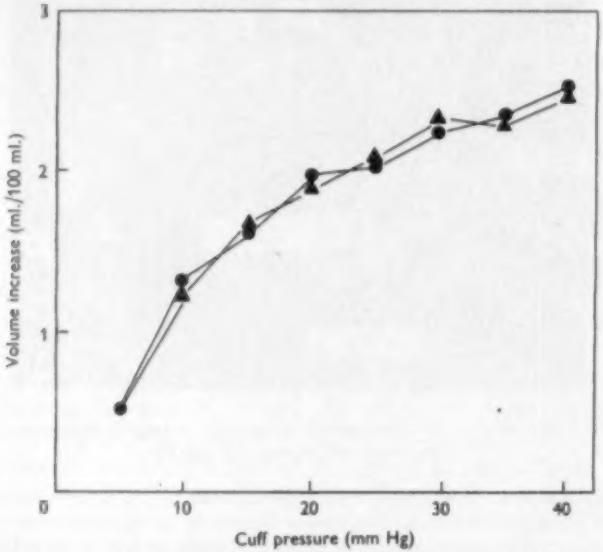

Fig. 2. Pressure–Volume curves obtained on a resting forearm by (▲) the 'step-down' method, and (●) the 'step-up' method.

Second series of experiments

In a second series of experiments, records were made: (1) during overventilation with air; (2) at rest; (3) while breathing through a tubular resistance attached to the mouth-piece. The order of these was reversed in alternate experiments. The rhythm of breathing during overventilation was regulated by a metronome at 23 breaths /min, and the average minute volume was 27 l. When breathing to the metronome at the same rate with resistance to inspiration and expiration, the average minute volume was 7 l.

Mouth-piece pressures were measured by a capacitance manometer attached to the mouth-piece, and during normal breathing were 0·7 mm Hg above and below atmospheric pressure. Overventilation caused changes of mouth-piece pressure of 1·5 mm Hg above and below atmospheric pressure, and breathing with resistance 4·0 mm. Hg above and below atmospheric pressure.

Fifty experiments were carried out on eight subjects.

RESULTS

Readings of volume changes were measured on the tracings as the vertical height of the end of each step level above the base line or volume at zero congesting pressure. A normal record of volume changes is shown in Fig. 3, and is compared with a superimposed record obtained during voluntary overventilation with 4% carbon dioxide. Pressure–volume curves constructed from these records are shown in Fig. 4. The chosen index of distensibility was the change in volume (ml./100 ml. tissue) caused by reducing the cuff pressure from 25 to 10 mm Hg (V_{10}^{25}). This index

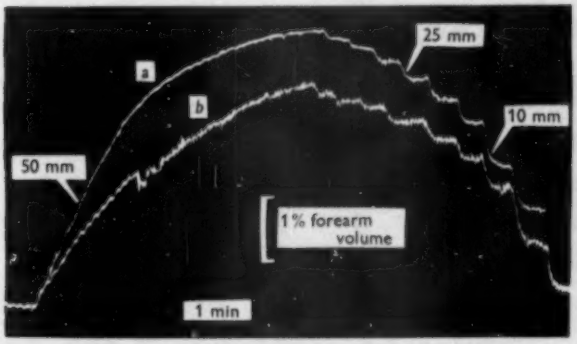

Fig. 3. Records of forearm volume changes (*a*) during rest (*b*) during overventilation with 4% CO_2. The apparently diminished blood flow during overventilation with 4% CO_2 is not a typical finding.

was selected because of lack of confidence in the efficiency of venous congestion at very low cuff pressures, and the reduction in gradient of the pressure–volume curves above 25 mm Hg. The mean values of V_{10}^{25}, obtained from ten resting tracings in each of the sixteen subjects, are shown in Table 1. This table also shows, for each stimulus, the change of V_{10}^{25} from the mean resting value in each subject.

First series of experiments

Effect of overventilation with air. A mean decrease of 0·184 from the normal V_{10}^{25} was found, this being a decrease of 12·3% from the average of all normal values. This result is significant at the 5% level ($0{\cdot}01 > P > 0{\cdot}001$).

Effect of overventilation with 4% CO_2. A mean decrease of 0·181 from the normal V_{10}^{25} was found, this being a decrease of 12·1% from the average of all normal values. This result is significant at the 5% level ($0{\cdot}001 > P$).

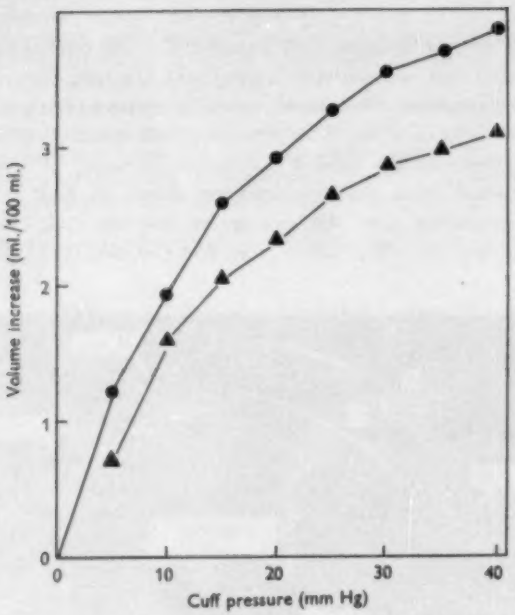

Fig. 4. Pressure–volume curves of forearm vessels, obtained during rest (●), and during overventilation with 4% CO_2 (▲).

TABLE 1

Subject	V_{10}^{20} normal mean	Change of V_{10}^{20} from normal (ml./100 ml. tissue) Overventilation with air	Overventilation with 4% CO_2	Breathing 9% CO_2
D.E.S.	1·18	−0·05	−0·18	−0·18
M.C.G.	1·85	−0·22	−0·41	−0·10
J.S.G.	1·62	−0·31	−0·31	0
E.C.	1·73	−0·37	−0·16	−0·37
D.H.	1·61	+0·10	−0·40	−0·25
P.F.	1·34	−0·28	−0·32	−0·32
W.M.	1·58	−0·47	−0·03	−0·27
P.H.	1·90	+0·02	−0·22	+0·02
T.A.	1·74	−0·38	−0·14	−0·22
J.C.	1·11	−0·37	−0·12	−0·12
J.D.	1·10	+0·10	−0·06	−0·02
J.T.	1·38	−0·45	−0·25	−0·58
E.M.	1·34	−0·08	−0·20	+0·26
R.B.	1·25	+0·05	0	0
A.O.	2·04	−0·04	−0·11	−0·25
L.E.E.	1·16	−0·20	+0·02	−0·20
Mean	1·494	−0·184	−0·181	−0·163
Significance of change		$t = 3·7$, $0·01 > P$	$t = 5·6$, $0·001 > P$	$t = 3·4$, $0·01 > P$

Effect of administration of 9% CO_2. A mean decrease of 0·163 from the normal V_{10}^{25} was found, this being a decrease of 10·9% from the average of all normal values. This result is also significant ($0{\cdot}01 > P > 0{\cdot}001$). There is a striking similarity in the averaged results of these first three stimuli.

Second series of experiments

The values of V_{10}^{25} obtained during rest, overventilation, and when breathing with resistance to air flow are shown in Table 2.

Overventilation with air. There was a mean decrease of 0·117 from the resting value of the index of distensibility. This decrease is significant at the 5% level ($0{\cdot}001 > P$).

TABLE 2. Values of the index of distensibility (V_{10}^{25}) obtained in fifty experiments on eight subjects

Rest	Over-ventilation	Resistance to breathing	Rest	Over-ventilation	Resistance to breathing
0·82	1·00	0·98	1·67	1·40	1·83
0·82	0·93	0·87	1·56	1·78	1·51
0·98	0·82	0·98	1·67	1·67	1·94
0·98	0·65	1·09	2·37	1·94	1·99
0·87	0·82	0·87	2·15	1·78	1·83
1·09	0·87	0·76	1·72	1·67	1·94
0·82	0·98	0·87	2·58	1·88	2·15
0·82	0·71	0·98	2·21	1·96	1·96
0·82	0·72	0·82	1·78	1·78	1·90
0·82	0·82	0·82	2·02	1·90	1·96
0·98	0·92	0·87	2·33	2·27	1·96
0·87	0·82	1·06	1·78	1·47	1·54
0·62	0·82	0·92	2·31	1·96	1·78
1·31	0·86	1·37	1·90	2·21	2·33
1·54	1·31	1·66	1·53	1·64	1·85
1·54	1·71	1·77	1·80	1·48	2·17
1·54	1·14	1·37	1·75	1·64	1·48
1·54	1·14	1·31	2·01	1·43	1·80
1·31	1·37	1·54	1·48	1·43	1·22
1·54	1·43	1·60	1·75	1·43	1·75
1·60	1·60	1·83	2·01	1·75	1·85
1·66	1·49	1·66	1·85	2·06	1·48
1·71	1·66	1·43	2·01	2·01	2·12
1·77	1·60	1·83	2·12	2·17	2·22
1·54	1·54	1·89	1·99	1·78	2·26
		Means	1·563	1·446	1·559

Breathing through a resistance. There was a mean decrease of 0·004 from the resting value of the index of distensibility. This decrease is not significant at the 5% level ($P > 0{\cdot}9$). There is a significant difference ($0{\cdot}01 > P > 0{\cdot}001$) of 0·112 between the values of V_{10}^{25} obtained during overventilation and those obtained during breathing through a resistance.

DISCUSSION

The effective stimulus causing the reduction in vascular distensibility during overventilation is not a reduction in the blood tension of carbon dioxide, for overventilation with carbon dioxide mixtures, causing the alveolar concentration to remain almost unchanged or even to increase, has the same effect as overventilation with air, which causes the alveolar concentration to decrease. These findings agree with those of Eckstein *et al.* (1958).

The effective stimulus is apparently not a pressure disturbance during overventilation. Breathing at normal depth through a resistance, which caused cyclical changes of mouth-piece pressure much greater than those observed during overventilation, brought about no change in distensibility. This observation is not conclusive, however, because it is possible that somewhere in the chest overventilation may cause greater pressure changes than does breathing through a resistance.

Leaving aside the last possibility, the common factor in those experiments which caused a reduction of distensibility was the greatly increased volume of tidal air. It seems probable that a receptor, stimulated either directly by the tidal excursion or by some consequence of it, is responsible for initiating the change in peripheral vascular distensibility.

A factor of emotional stress may enter into the application of these stimuli. It has been observed by Duggan *et al.* (1953) that the reduction of venous distensibility due to severe local cooling by immersion of the hand in iced water can be produced in some subjects merely by approaching with a beaker of iced water. Apprehension preceding the breathing of 9% carbon dioxide has been seen to cause a marked increase in forearm blood flow; it may also be a contributing factor in the observed distensibility changes. However, there is no reason to suppose that emotional stress with this degree of overventilation was greater than the emotional stress of breathing air through a resistance.

SUMMARY

1. A simple venous congestion method is described for obtaining pressure-volume relationships of the capacity vessels of the human forearm, at pressures within a normal physiological range.

2. The chosen index of distensibility was the change in volume (ml./100 ml. tissue) when the venous congesting pressure was reduced from 25 to 10 mm Hg.

3. In sixteen normal subjects overventilation with air, 4% CO_2, or 9% CO caused a significant decrease of about 12% in this index of distensibility.

4. Breathing through a resistance, a procedure designed to cause changes of intrathoracic pressure similar to those occurring in overventilation without the great increase of the respiratory minute volume, failed to cause significant changes in vascular distensibility.

5. It is suggested that some factor other than reduction of blood tension of CO_2 or the changes of intrathoracic pressure is mainly responsible for the reduction of vascular distensibility during overventilation.

I am indebted to Professor A. D. M. Greenfield for his assistance and advice during the investigation and in the preparation of this paper; also to the other members of the Department for their assistance.

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THE EFFECTS OF ACETYLCHOLINE ON THE VOLUME AND VASCULAR RESISTANCE OF THE DOG'S SPLEEN

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In a previous study of the effects of anticholinesterases on the spleen, Scott (1957) found that tetraethylpyrophosphate (TEPP) caused a small increase in volume after denervation of the spleen combined with bilateral adrenalectomy. Whether this response was the result of accumulation of acetylcholine or of a direct action of TEPP was not ascertained because passive effects due to alterations in arterial and venous pressures could not be ruled out.

Although the effects of acetylcholine on the spleen have been investigated previously by numerous workers, the results have been variable. In the dog, cat and rabbit intravenous injection of acetylcholine caused either a decrease in volume of the spleen (Ferguson, Ivy & Greengard, 1936), a decrease followed by an increase (Hunt, 1918; Gotsev, 1936) or only an increase in volume (Bacq & Fredericq, 1935). Hunt (1918) considered that the initial diminution in splenic volume produced by acetylcholine was a passive vascular effect due to the fall in systemic blood pressure, and that the subsequent increase in volume was due to a direct action of the drug on the muscular capsule of the spleen. In the view of Bacq & Fredericq (1935) the increase in volume of the spleen was due to vasodilatation within the organ. Studies on isolated strips of spleen *in vitro* indicate that acetylcholine causes contraction, not relaxation (Fredericq, 1929; Vairel, 1933; Saad, 1935; Ferguson *et al.* 1936).

The interpretation of changes in volume of the spleen occurring as a result of *intravenous* injections of a drug is often difficult because, apart from a direct effect on the organ, there are a number of other mechanisms by which the response may be brought about. First, the observed change in volume may be passive through an alteration in either arterial or portal venous pressure. Secondly, it may be the result of nervous influences on the spleen either by an action of the drug on the nervous system or reflexly through a change in arterial blood pressure. Thus Farber (1936) showed

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that the intravenous injection of acetylcholine in a dog whose spleen was isolated from the circulation and perfused by a second dog caused a diminution in volume of the organ, which could only have been brought about through the nerves to the spleen. Thirdly, the response of the spleen may be due to increased secretion of suprarenal medullary hormones.

The present study was undertaken first to discover the direct effect of acetylcholine on the spleen and secondly to investigate the mechanisms by which the responses were brought about. To exclude nervous and humoral effects the spleen was isolated and perfused with blood and injections were made into the splenic artery. This technique had the advantage that it enabled changes in vascular resistance to be measured simultaneously with changes in volume of the organ.

METHODS

Dogs, varying in weight from 10·2 to 18·6 kg, were anaesthetized with 0·25 ml./kg intravenously of a 1:1 mixture of Dial Compound (Ciba Laboratories Ltd, diallylbarbituric acid 0·1 g and urethane 0·4 g/ml.) and pentobarbitone sodium solution (Nembutal, Abbott Laboratories, Ltd, 60 mg/ml.) preceded by morphine hydrochloride (3 mg/kg subcutaneously). One animal was given chloralose (0·1 g/kg intravenously) after premedication with morphine (3 mg/kg subcutaneously).

In most experiments the spleen was freed from its nervous connexions by removal from the abdomen; it was perfused from a femoral artery of the same animal. The vascular connexions of the spleen with the omentum, pancreas and stomach were divided between ligatures. The splenic artery was tied close to its origin from the coeliac artery and cannulated towards the spleen; the splenic vein was likewise cannulated. The remaining tissue in the splenic pedicle was then divided between ligatures, and the spleen was removed from the abdomen and placed in a Perspex plethysmograph. The method used for perfusion of the spleen is shown in Fig. 1. Blood from a femoral artery passed through the flowmeter *a* into the splenic artery *b*. The blood was returned to the animal from the splenic vein *c* via a femoral vein. The arterial inflow pressure to the spleen was measured from a T-piece in the arterial tubing by a mercury manometer. The splenic vein pressure was measured from the venous tubing with a vertical saline manometer, the open end of which was connected to a small volume recorder. The flowmeter used for measuring blood flow was a rotameter of the Shipley & Wilson (1951) type, in which were incorporated the modifications described by Bell (1954). Blood flow was recorded continuously on the kymograph by means of a direct recording milliammeter. The instrument was calibrated at the end of each experiment as described by Daly (1957).

In all these experiments coagulation of the blood was prevented by heparin (Liquemin, Roche Products, Ltd, 7–21 mg/kg). Unless otherwise stated, injections of drugs were made into the splenic arterial tubing distal to the rotameter. Control injections were made in nearly every experiment with a solution of sodium chloride, 0·9 g/100 ml., and were without effect. The volume of the injected fluid did not exceed 0·2 ml.

The plethysmograph was filled with liquid paraffin and rested on an electric heating pad to maintain the temperature of the fluid at 37–39° C. Changes in volume of the spleen were measured by means of a Krogh type of volume recorder connected to the plethysmograph.

In a few experiments the splenic nerve was placed on platinum wire electrodes inside the plethysmograph. An Attree (1950) electronic stimulator giving independent control of the voltage, frequency and duration of the rectangular pulses was used.

In one experiment changes in volume of the naturally perfused spleen with the splenic nerve intact were measured using the plethysmograph. To mobilize the spleen sufficiently to enable it to be put into the plethysmograph its vascular connexions with the stomach were severed. In this experiment no heparin was given. Drugs were injected via an indwelling needle in the splenic artery.

In three experiments the animals were treated with reserpine (Serpasil, Ciba) to deplete the stores of catechol amines. Two doses of 0·1–0·2 mg/kg, intravenously or intraperitoneally, were given on successive days, the acute experiment being performed the day after the second injection.

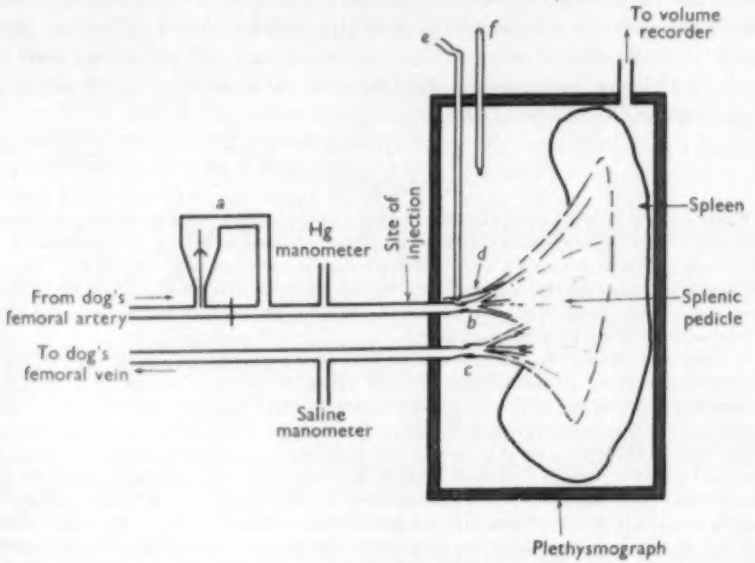

Fig. 1. Diagram showing the method of perfusing the spleen at a constant head of pressure. *a*, rotameter; *b*, splenic artery; *c*, splenic vein; *d*, splenic nerve; *e*, stimulating electrodes; *f*, thermometer. For further details see text.

The following drugs were used: acetylcholine chloride (Roche Products, Ltd), atropine sulphate (British Drug Houses, Ltd), hexamethonium bromide ('Vegolysen', May and Baker, Ltd), eserine sulphate (British Drug Houses, Ltd), tetraethylpyrophosphate (Albright and Wilson), dibenzyline hydrochloride (Smith, Kline and French), dibenamine (Light and Co. Ltd), nicotine acid tartrate (British Drug Houses, Ltd) and 1:1-dimethyl-4-phenylpiperazinium iodide (DMPP, Pharmacia).

Degenerative section of the nerves to the spleen

At a preliminary operation carried out under pentobarbitone anaesthesia with full aseptic precautions, the spleen was denervated by cutting all the attachments to neighbouring organs and by dividing the splenic nerve at the level of the origin of the splenic artery. Thus the spleen was only connected by the splenic artery and vein, which were stripped of the surrounding nerve plexus as far as possible. The acute experiment was performed 8–10 days later.

RESULTS

Effects of acetylcholine

Splenic volume. The response of the isolated perfused spleen to acetylcholine injected into the splenic arterial tubing depended on the dose. In twelve experiments the smallest effective dose, which varied from 0·01 to 5 μg in different experiments, always caused a small increase in splenic volume, which rarely exceeded 5 ml. This response occurred within 2–3 sec of injection and usually lasted not more than 2 min, although occasionally a response lasting up to 5 min was observed. The same response was produced in the naturally perfused innervated spleen. Larger doses of acetylcholine, 5–25 μg, produced an increase in splenic volume which was followed by a decrease; a second increase in volume then sometimes occurred. With still larger doses, only a decrease in volume took place. Compared with the increases in volume evoked by small doses of acetylcholine, the decreases were large, frequently 20–30 ml. Typical responses are shown in Figs. 2 and 3*A*.

All these responses occurred in the adrenalectomized preparation and without change in splenic arterial pressure. A rise in venous pressure of 1–2 cm saline sometimes occurred during contraction of the spleen. This was probably due to a temporary increase in splenic venous blood flow resulting from emptying of the blood stores of the organ.

Vascular resistance. In eleven experiments acetylcholine invariably caused an increase in splenic arterial blood flow which was sometimes preceded by a momentary decrease. Since the splenic arterial and venous pressures remained constant, these changes in blood flow indicate a similar directional change in splenic vascular resistance. The vasodilator response usually lasted up to 2 min, although occasionally the increase in blood flow was prolonged, as in the experiment illustrated by Fig. 3*A*. These vascular responses occurred with doses of acetylcholine varying from 0·05 to 100 μg and independently of the changes in volume of the spleen. It was noted, however, that in those tests in which the acetylcholine vasodilator response was prolonged, the increase in volume of the spleen also persisted (Fig. 3*A*).

Atropinized spleens. Farber (1936) found that acetylcholine in doses of 0·5–4 mg injected into the splenic artery of the atropinized dog caused contraction of the spleen. In our experiments atropine, in doses of 2–5 mg (0·16–0·3 mg/kg) intravenously, invariably abolished the increase in splenic volume evoked by the small doses of acetylcholine. When the response to acetylcholine was diphasic, atropine also reduced or abolished the decrease in volume (Fig. 3*A*, *B*). If the dose of acetylcholine was now increased a reduction in volume invariably reappeared at a dose level 1·5–10 times that required to evoke the same response before atropine

(eight experiments). This effect, occurring in atropinized preparations, did not appear to be caused by the larger doses of acetylcholine overcoming the atropine block, because it could still be obtained after a second dose of atropine, although occasionally it was then slightly reduced (Fig. 3*B*, *C*).

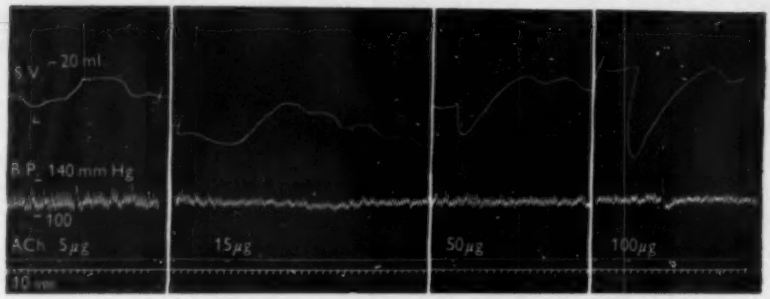

Fig. 2. Dog, *m*, 14·9 kg. Dial-Nembutal. Innervated spleen with natural blood supply. The effects of acetylcholine in doses of 5, 15, 50 and 100 μg injected into the splenic artery. In this and in subsequent figures: S.V. = splenic volume (increase in volume upwards); S.V.P. = splenic venous pressure; S.A. flow = splenic arterial blood flow; B.P. = femoral arterial blood pressure.

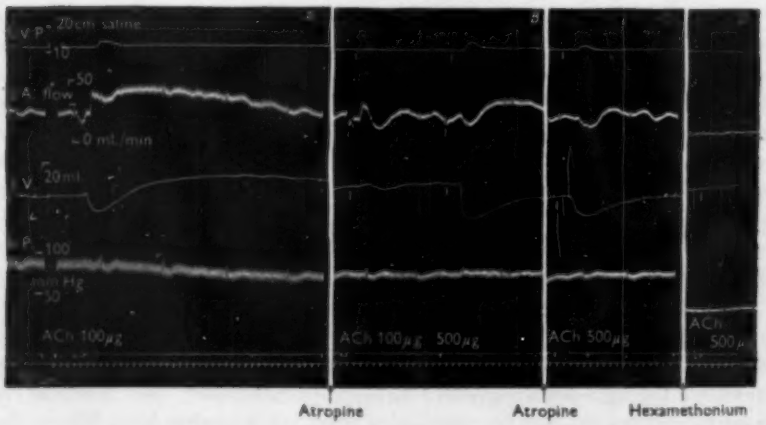

Fig. 3. Dog, *f*, 12·0 kg. Dial-Nembutal. Perfusion of the isolated spleen. *A–D* show the effects of close arterial injections of acetylcholine into the spleen. Between *A* and *B*, atropine 2 mg injected intravenously. Between *B* and *C*, a further 2 mg atropine. Between *C* and *D* hexamethonium, 10 mg/kg, intravenously.

The effect of atropine on the acetylcholine vasodilator response was variable. Usually the response was abolished, but occasionally acetylcholine initiated rhythmic variations in blood flow (Fig. 3*B*, *C*). In other experiments acetylcholine caused vasoconstriction in atropinized preparations. In this connexion, Hunt (1918) found that the increase in the venous outflow caused by acetylcholine in the isolated cat's spleen perfused at a constant head of pressure was abolished by atropine; large doses of acetylcholine after atropine caused a reduction in outflow.

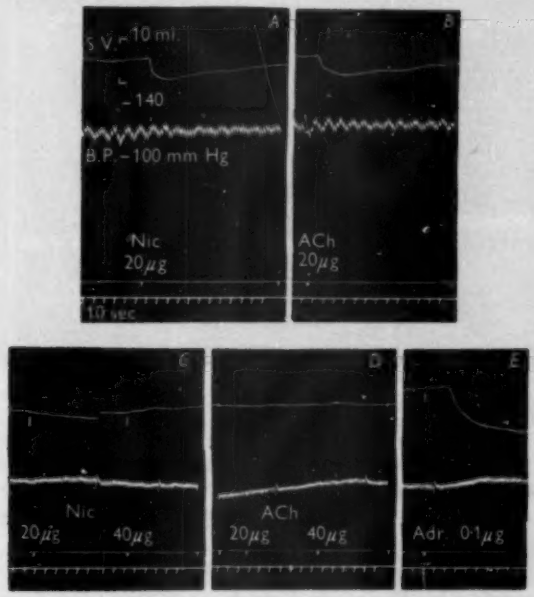

Fig. 4. Dog, *f*, 18·0 kg. Dial-Nembutal. Bilaterally adrenalectomized. Perfusion of the isolated spleen. Atropine 4 mg before recording begun. *A*, nicotine, 20 μg. *B*, acetylcholine, 20 μg. Between *B* and *C*, hexamethonium, 10 mg/kg, intravenously. *C*, nicotine, 20 and 40 μg. *D*, acetylcholine, 20 and 40 μg. *E*, adrenaline, 0·1 μg. All injections not specified given into the splenic arterial tubing.

For reasons which will be discussed below, we attribute the small increases in volume of the non-atropinized spleen evoked by acetylcholine, not to an action on the capsule, but to a passive effect of vasodilatation in the organ. Our finding that acetylcholine evoked large reductions in volume is in keeping with the results of previous workers who found that acetylcholine caused contraction of isolated strips of the capsule. Our results suggest that there are two mechanisms by which acetylcholine

causes contraction of the spleen; one of these is abolished by atropine and is therefore a muscarine-like action, the other is a nicotine-like effect because it is practically unaffected by atropine. This latter response can be mimicked by nicotine, 15–20 μg, and by DMPP, 2–10 μg (Figs. 4*A*, 5*C*).

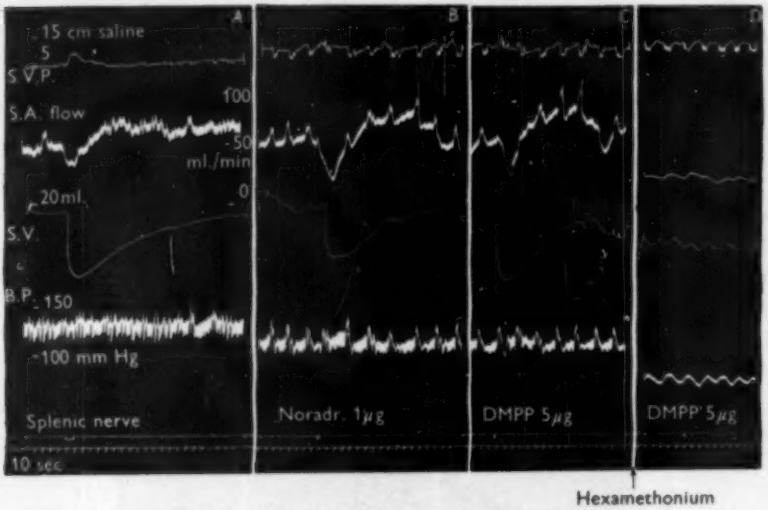

Fig. 5. Dog, *m*, 11·2 kg. Morphine-chloralose. Perfusion of the isolated spleen. Atropine, 6 mg, before recording begun. *A*, electrical stimulation of the splenic nerve, 8 V, 0·2 msec, 100 c/s. *B*, noradrenaline, 1 μg. Between *C* and *D*, hexamethonium, 10 mg/kg, intravenously. *C* and *D*, DMPP, 5 μg. All injections not specified given into splenic arterial tubing. Initial volume of spleen about 150 ml.

The effect of a ganglionic-blocking agent

It was reported by Fotino & Stoiculesco (1956) that the acetylcholine contraction of the spleen was unaffected by hexamethonium. Our results indicate that the effect is dependent on whether or not atropine was given previously.

Non-atropinized spleens. The effect of hexamethonium, 10 mg/kg intravenously, was tested in three preparations. It was found that the increase in volume of the spleen and the vasodilatation produced by threshold doses of acetylcholine were unaffected by hexamethonium. The contraction of the spleen with larger doses also persisted. Since these effects were abolished by a subsequent injection of atropine, these findings provide further evidence that the contraction of the spleen is in part due to a muscarine-like effect of acetylcholine, whose site of action must be peripheral to the ganglia.

Atropinized spleens. Hexamethonium invariably abolished the contraction of the spleen evoked by acetylcholine, nicotine, and by DMPP. The effects are illustrated by Figs. 3*C*, *D*, 4 and 5*C*, *D*. The increase in splenic vascular resistance produced by these substances was also abolished (Fig. 5*C*, *D*). On the other hand, hexamethonium had no appreciable effect on the contraction caused by splenic nerve stimulation.

Effect of anticholinesterases

In two atropinized preparations the contraction of the spleen produced by acetylcholine was potentiated by eserine in doses of 0·06 and 0·07 mg/kg intravenously respectively. A similar result was obtained in two other atropinized preparations with TEPP, 0·1 mg/kg intravenously.

Effect of anti-adrenaline drugs

In two atropinized preparations the effect of acetylcholine and nicotine was tested before and after intravenous injection of dibenzyline in doses of 10 and 15 mg/kg respectively. In both experiments the contraction of the spleen was considerably reduced. The small residual effect which occurred may be due to dibenzyline incompletely blocking transmission at sympathetic post-ganglionic nerve endings. This view is supported by the fact that these doses of dibenzyline did not completely abolish the contraction of the spleen evoked by close arterial injection of noradrenaline or by electrical stimulation of the splenic nerve. The normal responses to noradrenaline, and splenic nerve stimulation are shown in Fig. 5*A*, *B*. In one further experiment in which the spleen was naturally perfused, thereby obviating the need to give heparin, dibenamine abolished the contraction of the atropinized spleen produced by 150 μg acetylcholine injected into the splenic artery (Fig. 6). These results indicate therefore that the effect of nicotine and the nicotine-like effect of acetylcholine are reduced or abolished by agents opposing the effects of adrenaline and noradrenaline.

Effect of chronic denervation of the spleen

Since the majority of sympathetic preganglionic fibres to the spleen relay in the coeliac plexus, division of the splenic nerve at the level of the origin of the splenic artery would result in degeneration of post-ganglionic fibres with their cells in this plexus. Aberrant ganglion cells situated along the course of the splenic artery would not, however, be affected (Kuntz & Jacobs, 1955).

In three atropinized spleens which had been denervated at a previous operation (see Methods), neither acetylcholine, nicotine nor DMPP in doses up to 50 μg had any effect on the volume of the spleen. In one

experiment large doses of acetylcholine of 0·5 and 1 mg were tested and these caused small reductions of about 2 ml. in the volume of the spleen followed by an increase. The absence of any response to injection of these drugs was not due to the preparation being unresponsive to drugs, because 0·5 μg doses of noradrenaline had profound effects.

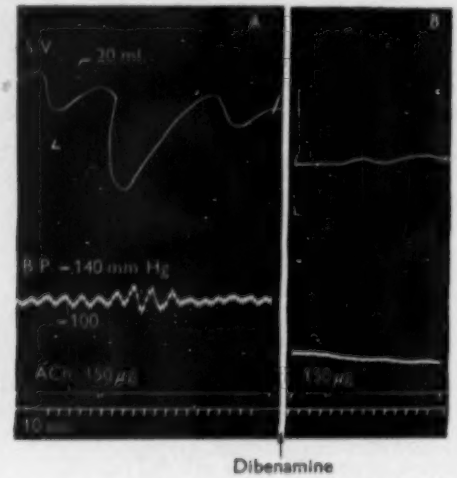

Fig. 6. Dog, *m*, 14·9 kg. Dial-Nembutal. Innervated spleen with natural blood supply. Atropine 4 mg before recording begun. *A* and *B* show the effects of close arterial injection of acetylcholine, 150 μg, into the spleen. Between *A* and *B*, dibenamine, 400 mg, intravenously. A period of 1 hr 15 min elapsed between the injections of dibenamine and acetylcholine in *B*.

Effect of reserpine

The response of the atropinized spleen to acetylcholine, in doses of 25–150 μg, was reduced but not abolished by reserpine. In two of these experiments the spleen failed to respond to nerve stimulation, but in the third experiment stimulation of the splenic nerve caused a small diminution in volume, indicating incomplete depletion of the stores of catechol amines. It is of interest to note that Burn & Rand (1960) were rarely able to abolish the contraction of the spleen of the cat in response to stimulation of the splenic nerve by treatment with reserpine.

DISCUSSION

Our results have shown that the smallest effective dose of acetylcholine almost invariably causes an increase in volume of the spleen; with larger doses this is followed by contraction, and with still larger doses only con-

traction takes place. These effects occur not only in acutely denervated spleens, but also in the naturally perfused, innervated spleen. Since all injections were made into the splenic artery the responses must be the result of a direct effect on the spleen or on structures in the splenic pedicle.

The increase in splenic volume with small doses of acetylcholine may be due to a passive distension of the organ through dilatation of its blood vessels, since the increases in volume were always accompanied by vasodilatation, and the two effects occurred simultaneously. Moreover, both the vasodilator response and the increase in splenic volume caused by acetylcholine were unaffected by hexamethonium but were abolished by atropine. Finally, previous workers have shown that in isolated strips of spleen, acetylcholine causes contraction, not relaxation (Fredericq, 1929; Vairel, 1933; Saad, 1935; Ferguson *et al.* 1936; Brandon & Rand, 1961).

Bacq & Fredericq (1935) were also of the opinion that the increase in volume of the spleen evoked by acetylcholine was due to vasodilatation in the organ. Grindlay, Herrick & Baldes (1939) made simultaneous measurements of splenic arterial and venous blood flow and of splenic volume in dogs, and presented evidence which suggested that the rhythmic volume changes were caused, not by alterations in the tone of the capsule, but by variations in blood flow through the spleen.

The contraction of the spleen produced by larger doses of acetylcholine is due to two different actions: (1) a muscarine-like action on the capsule of the spleen, which is abolished by atropine (Dale, 1914) but unaffected by hexamethonium; (2) a nicotine-like effect seen in the atropinized spleen, which can be mimicked by nicotine and DMPP, and is abolished by hexamethonium. These nicotine-like substances also mimic the effects produced by splenic nerve stimulation and by adrenaline and noradrenaline, and since they become ineffective after chronic denervation of the spleen, they must act somewhere on the nerve supply to the organ. It seems unlikely that the vagus plays any part, because there appears to be no evidence for any definite action of the vagus nerve on the spleen (Schäfer & Moore, 1896; Masuda, 1927). Furthermore, Utterback (1944) found no change in the number of myelinated and non-myelinated fibres in the splenic nerve after degenerative section of both vagus nerves below the level of the diaphragm. Presumably, therefore, these substances act through the sympathetic nervous system.

There are four possible mechanisms by which nicotine-like substances may cause contraction of the spleen, namely stimulation of (1) sympathetic ganglion cells; (2) sympathetic post-ganglionic nerve fibres or endings in continuity; (3) sensory nerve endings, with the initiation of an axon reflex; and (4) a release of adrenaline or noradrenaline from stores beyond the nerve endings.

There is some evidence in favour of the view that the nicotine-like action of acetylcholine on the spleen is the result of stimulation of peripheral ganglion cells situated in the vascular bed of the splenic artery. Ambache (1951) has suggested that the inhibitory action of nicotine on the intestine might be due to stimulation of local adrenergic ganglion cells, since it was abolished by hexamethonium and by large doses of nicotine. We have shown that, in atropinized spleen preparations, (1) the acetylcholine contraction of the spleen is potentiated by anticholinesterases and is abolished by hexamethonium; (2) ganglion-stimulating drugs such as nicotine and DMPP cause contraction of the spleen, which is also abolished by hexamethonium, and by anti-adrenaline drugs such as dibenzyline or dibenamine. On the other hand, this view which would ascribe the nicotine-like effect of acetylcholine on the spleen to an action on ganglia situated in either the spleen or splenic pedicle is contrary to the generally held concepts of the organization of the sympathetic nervous system, which assume that all preganglionic sympathetic fibres traversing the coeliac plexus relay in this plexus; the post-ganglionic fibres issuing from it then proceed to the organ which they innervate. With regard to the spleen, this view is supported by the work of Glaser (1928), Riegele (1929) and of Utterback (1944). Langley (1896), on the other hand, found in the cat small ganglia along the course of nerve strands from the solar ganglia. He states, 'I have no doubt that some of the splanchnic fibres run on past the solar ganglia to be connected with more peripheral ganglia, just as some fibres run through the inferior mesenteric ganglia on their way to more peripheral ganglia'. Evidence for sympathetic nerve fibres passing through the coeliac ganglion and relaying more peripherally was also obtained by Babkin, Hebb & Sergeyeva (1939). More recently, Kuntz & Jacobs (1955) have demonstrated by histological methods the presence of ganglia along the course of the splenic artery in the cat and rat, although not in man. At our request, the late Dr A. Kuntz very kindly examined the nerves accompanying the splenic artery in the dog for ganglion cells. He reported as follows:

> The first one examined showed a small ganglion in the splenic plexus in which we counted forty-seven ganglion cells. This seemed rather meagre, so we examined two more without finding any ganglion cells. Our findings in the first dog show that ganglia may occur in the nerves along the splenic artery. Finding none in the next two dogs was rather disappointing, but I do not take this to mean that ganglion cells occur only very rarely in the splenic nerves in the dog.

We regard it as most unlikely, however, that the mechanism of action of nicotine-like substances is on ganglion cells in the spleen, for two reasons. First, although the histological evidence suggests that ganglion cells are not to be found consistently in the dog's splenic nerve, nicotine-like substances regularly caused contraction of the spleen. Secondly, this

contraction could not be evoked after degeneration of the nerves to the spleen, a procedure which these ganglion cells would be expected to survive.

The second possibility, that nicotine-like substances may act on post-ganglionic nerves in continuity, is also unlikely because acetylcholine does not stimulate isolated frog's nerve (Lorente de Nó, 1944), and Brown & MacIntosh (1939) found that the intra-arterial injection of acetylcholine fails to set up action potentials in nerves.

The third possibility is suggested by the fact that acetylcholine and nicotine-like substances can excite sensory nerve endings (see Gray & Diamond, 1957). The centripetal discharge of impulses evoked by acetylcholine is prevented by hexamethonium, and the sensory ending in general shows many of the pharmacological properties of a sympathetic ganglion cell. The acetylcholine contraction of the spleen might be due to such an axon reflex set up by the excitation of sensory nerve endings, but there are, of course, difficulties in accepting the existence of such reflexes in the spleen. It implies either that sensory receptors form an integral part of the peripheral sympathetic system or that there exist in the spleen sensory axons, a collateral branch of which supplies the capsule of the organ with adrenergic fibres. The only means of deciding between these two possibilities would be to perform a chronic section of posterior nerve roots of such an extent that the spleen is deprived of all afferent connexions.

Axon reflexes involving post-ganglionic (non-sensory) branching collateral fibres have been postulated previously to explain the action of certain nicotine-like substances (Coon & Rothman, 1940; Ambache & Robertson, 1953). Their effects occur in atropinized preparations and are abolished by hexamethonium and by chronic denervation of the organ.

The fourth possibility is that acetylcholine causes contraction of the atropinized spleen through the release of adrenaline and noradrenaline from stores surrounding the sympathetic nerve endings (see Burn & Rand, 1959*b*, 1960). Our results have shown that the contraction produced by acetylcholine is reduced or abolished after degeneration of the splenic nerve and after treatment with reserpine. These procedures also considerably reduce the noradrenaline content of the spleen (von Euler & Purkhold, 1951; Burn & Rand, 1959*a*; Brandon & Rand, 1961). Our finding that the response to acetylcholine is abolished by dibenamine and by dibenzyline is also in keeping with the view that the nicotine-like effects of acetylcholine on the spleen are due to the release of noradrenaline. Brandon & Rand (1961), using the cat's spleen, have obtained similar results. The site of action of acetylcholine and nicotine is at present uncertain. Gillespie & Mackenna (1960) have tentatively suggested an action on some form of terminal sympathetic nerve network intervening between the sympathetic

nerves and the smooth muscle. Our own experiments provide no further information, and clearly further work is required to establish this point.

SUMMARY

1. Close arterial injections have been made into the dog's spleen isolated and perfused with blood at a constant head of pressure. Splenic arterial blood flow was measured continuously by means of a rotameter.

2. The smallest effective doses (0·01–5 μg) of acetylcholine caused a small increase in volume of the spleen and a decrease in vascular resistance. These effects were abolished by atropine. The increase in volume of the spleen is attributed to a passive effect of dilatation of the splenic blood vessels.

3. Larger doses (5–100 μg) of acetylcholine caused contraction of the spleen and this response occurred in atropinized preparations. Since nicotine had a similar effect, this action of acetylcholine is due to its nicotine-like properties. It was abolished by hexamethonium.

4. When the nicotine-like action of acetylcholine was prevented by hexamethonium in non-atropinized preparations, injection of acetylcholine still caused contraction of the spleen and this response was now abolished by atropine. This suggests that acetylcholine also exerts a muscarine-like action directly on the capsule of the spleen.

5. The contraction of the atropinized spleen by acetylcholine or nicotine is abolished by sympatholytic drugs and by degenerative section of the nerves to the spleen, and is reduced by treatment with reserpine.

6. The possibility that the nicotine-like actions of acetylcholine are due to the release of noradrenaline is discussed.

We wish to express our thanks to Mr D. R. Bacon for technical assistance and to Drs Brandon and Rand for allowing us to read the manuscript of their paper before publication. This work was supported in part by a grant from the Royal Society to one of us (M. de B. D.).

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THE IMMEDIATE EFFECTS OF ELASTIC LOADS ON THE BREATHING OF MAN

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Most accounts of the control of breathing imply that the variations in contraction of the respiratory muscles required to produce the movements of breathing are dictated by the respiratory centre in the medulla. The activity of this centre and the behaviour of the muscles are generally regarded as being subject to the two major influences. First, the chemical state of the blood governs the level of centre discharge in such a way as to control the ventilation. Secondly, the activity of pulmonary stretch receptors transmitted by the vagi governs the depth of each breath by inhibiting the inspiratory centre when the lungs are distended to a certain volume.

The behaviour of the respiratory muscles is probably subject to central and reflex influences, other than those affecting the respiratory centre, which may vary throughout the respiratory cycle. There has, however, been no demonstration of such mechanisms, nor any experimental examination of their role in breathing.

During a study of the ability of conscious human subjects to detect sudden added loads to breathing (Campbell, Freedman, Smith & Taylor, 1961) the addition of the loads affected the tidal volume in a manner which was difficult to reconcile with the known mechanisms controlling breathing. The work reported in this paper was designed to study this behaviour in more detail.

METHODS

Elastic loading. The elastic loads were provided by rigid closed containers in which changes in pressure are functions of the changes in gas content according to Boyle's Law. The smaller the container, the greater is the change in pressure for a given change in gas content, i.e. the greater is the load. Elastic loads were employed in this study because, at the ends of inspiration and expiration when there is no air flow, and the dynamic non-elastic loads are absent, changes in tension developed by the inspiratory muscles can be inferred from observed changes in the volume of the chest. The loads have been described by their 'elastance' (Bayliss & Robertson, 1939), that is to say, the change in pressure produced by the addition or removal of gas of unit volume at ambient pressure (cm H_2O/l.).

The apparatus (Figure 1 and Table 1). A number of empty oil drums, varying in volume from 2·5 to 50 Imp. Gallons (11·4–227·5 l.), were connected in parallel by wide-bore (2·5 cm i.d.) thick-walled rubber tubing. The number of drums in connexion with the main circuit could

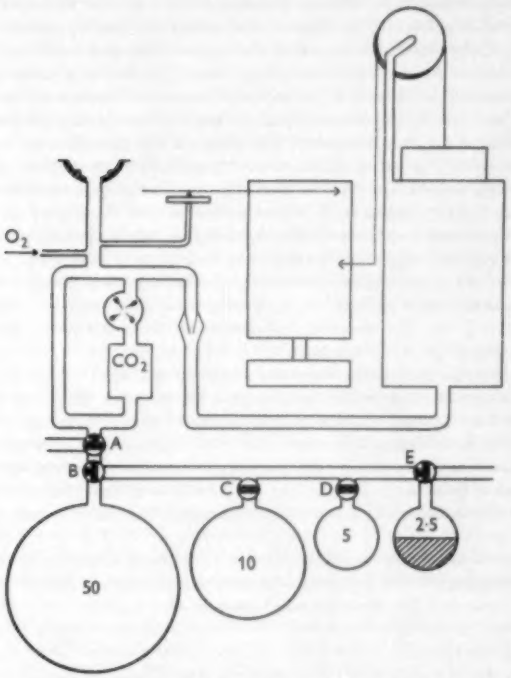

Fig. 1. Arrangement of the apparatus (diagrammatic, for description see text). The drums are shown at the bottom, the numbers referring to their volume in Imperial Gallons. Tap A was used to switch the subject into the drum circuit; tap B to switch the subject from the control load (50) to the others; taps C, D, E to provide the requisite added loads; tap E was also used to maintain the drums at atmospheric pressure between applications. CO_2, CO_2 absorber.

TABLE 1

Volume of drum* (Imp. Gallons)	Volume of drum* (l.)	Volume of rest of circuit to valve (l.)	Elastance (cm H_2O/l.) Calculated	Elastance (cm H_2O/l.) Observed
50	227·5	1·5	4·45	4·75
15	68·0	1·6	14·9	14·0
5	22·8	1·5	45·6	36·0
(2·5)	(11·4)	1·4	(91·5)	46·0

Dimensions of the drums

*The values quoted for volume are the trade descriptions of the drums. 15 gallons were obtained by using three 5-gallon drums in parallel. The '2·5'-gallon drum was a 5-gallon drum approximately half-filled with water (water being added until an elastance of 46 cm H_2O/l. was obtained). An elastance of 23 cm H_2O/l. was produced when the 5- and 2·5-gallon drums were in parallel.

be adjusted by operating taps. The subject breathed from the drums through a circuit containing a circulating fan, a CO_2 absorber and a lead from an O_2 cylinder from which O_2 was supplied at a rate slightly in excess of the subject's oxygen consumption. In parallel with the CO_2 absorber there was an expiratory valve. The fall in pressure produced at the mouth-piece by inspiration closed the valve, and it remained closed until, towards the end of expiration, the pressure in the whole circuit exceeded the opening pressure of the valve and the back pressure of the spirometer. The effect of this arrangement was to produce a constant end-expiratory pressure in the whole circuit. Any air expired by the subject in excess of that inspired (plus the O_2 flow) was collected in the spirometer and recorded on a kymograph. The opening pressure of the valve varied less than $\pm 0{\cdot}2$ cm H_2O during an experiment. This variation can have little physiological effect. It caused no error, because all relevant pressure and volume deflexions were individually measured.

The pressure at the mouth-piece was measured with a rubber membrane manometer of 10 cm diameter and a lever writing on the kymograph. The response of this arrangement was faithful up to 2 c/s. The recorded deflexion was 1 cm/cm H_2O at ambient pressures, falling to a quarter of this at 20 cm H_2O.

Subjects. The conscious subjects were nine healthy men, aged 21–34. They were studied while reclining in an arm chair breathing through a mouth-piece. The anaesthetized subjects were two men and one woman, aged 29–46, who had no clinical or radiological evidence of lung disease. They were studied supine immediately before operation. After premedication with pethidine and hyoscine, anaesthesia was induced with sodium thiopentone and maintained with thiopentone or halothane. Respiratory paralysis was induced with succinylcholine, and a cuffed endotracheal tube was inserted through which the subject was connected to the circuit.

The measurement of thoracic elastance. The total thoracic elastance of the anaesthetized subjects was measured in the following way immediately after intubation, while the subjects were still paralysed. The pressure was increased and measured (P_1) in one of the drums of the circuit; the subject's endotracheal tube connexion was opened to the air for a few seconds to bring the pressure in the lungs to atmospheric pressure (P_0); the drum was then connected with the subject's lungs. The pressure was allowed to equilibrate and was measured (P_2) after 3–6 sec. The volume of air taken in by the subject's lungs (ΔV) was calculated, knowing the elastance of the drum, from $P_1 - P_2$. The change in pressure in the subject's lungs (ΔP) was $P_2 - P_0$; hence the elastance ($\Delta P/\Delta V$) could be derived. This method underestimates the volume of air entering the subject's chest (and thus overestimates the elastance) by a percentage of $100 \times (P_2 - P_0)/P_0$, where the pressures are in absolute units. As the final pressures (P_2) were always less than 10 cm H_2O above P_0 the error was less than 1 %. Attempts were made to measure the total thoracic elastance of the conscious subjects in several ways. None gave reliable results.

Calibration of added elastic loads. When the larger drums were in communication, the elastance of the circuit was close to that calculated from their volumes. However, as the volume in communication was reduced, the compliance of the rubber tubing of the circuit caused the true elastance to fall below that calculated. The elastance values attributed to the various combinations of drums used in the experiment were therefore directly measured by adding or withdrawing known volumes of air measured at ambient pressure, and measuring the pressure changes (Table 1).

Calculation of tidal volume. Knowledge of the elastance of the various circuit arrangements enabled tidal volumes to be calculated from the pressure record. Thus a change in pressure from 0 to -10 cm H_2O while inspiring from an elastance of 23 cm H_2O/l. implies a tidal volume of $(10 \div 23$ l.$) = 435$ ml. This technique involves a number of errors, none of which is important in the present study.

First, the method of calculation described above neglects the expansion of the gas in the lungs produced by the fall in pressure during inspiration. The error introduced by neglect of this factor depends upon the pressure change and the functional residual capacity (FRC)

of the lungs. If we assume a high normal value for the FRC (3 l.) and if we take the highest observed fall in pressure (30 cm H_2O) the maximum likely error would be an underestimation by 90 ml. in a tidal volume of 600 ml. at an elastance of 46 cm H_2O/l. If we assume an FRC of 2 l. and take an average pressure swing (11 cm H_2O) then the error would be 20 ml. in a tidal volume of 500 ml. at an elastance of 23 cm H_2O/l. We have not corrected for this factor, first because of lack of knowledge of the volume of gas in the lungs; secondly, because the error is small over the main range of observations; and thirdly, because the effect of taking it into account would only be to magnify the effects to be described.

Secondly, the method of calculation neglects the increase in volume of the gas removed from the drums as a result of the fall in its pressure. The error introduced, expressed as a percentage, is 100 × (barometric pressure − observed pressure) ÷ barometric pressure. As the difference between observed and barometric pressure never exceeded 30 cm H_2O the error is less than 3 % of the calculated tidal volume. We have not corrected for this factor because we consider it to be negligible and because the effect of taking it into account would only be to magnify the effects to be described.

Thirdly, the method of calculating tidal volume neglects the expansion of the air as it increases in temperature on passing from the circuit into the lungs. The temperature at the entry to the drums did not vary during a prolonged period of rebreathing. In the circuit closer to the subject it occasionally reached 2–3 °C above that of the room, and prevented the usual simple correction from ATPS to BTPS from being applied. For this reason, and because any correction would have a negligible effect on comparisons of tidal volume, we decided to ignore the influence of temperature.

Fourthly, the continuous feed of oxygen into the circuit might have caused errors had it been large or had it varied with the pressure fluctuations. The rate was monitored with a rotameter and varied very little during the pressure fluctuations. The excess inflow may have caused an underestimate of up to 3 ml. in some calculated tidal volumes. We consider this to be negligible.

Finally, there was a random error due to the accuracy of pressure recording. This error varied both with the pressure and the elastance. We consider the calculated tidal volumes, neglecting the systematic errors discussed above, are correct to ±2·5 %.

Procedure

Control periods were recorded while the subject was rebreathing from the largest drum (elastance 4·75 cm H_2O/l.) and the rate of addition of O_2 was adjusted until it was 50–100 ml./min greater than the subject's O_2 consumption, thus producing a downward slope on the record (Fig. 2). The required additional load was selected by operating the taps connecting the other drums and then, at the end of an expiration, tap B was turned to switch from the control elastance to the higher one. After five breaths, again at the end of expiration, the subject was returned to the control circuit. In six of the subjects (3 conscious and 3 anaesthetized) four added loads of 14, 23, 36 and 46 cm H_2O/l. were each presented six or eight times in a factorial manner in order to enable reproducibility of the responses to be assessed.

RESULTS

A typical record of the response of an anaesthetized subject to the application of each of the four different elastic loads is shown in Fig. 2. This record is representative of those obtained in all subjects.

The upper trace is the record of pressure changes at the mouth. The switching from the control system of containers (elastance 4·75 cm/l.) to smaller containers is indicated by the development of lower pressures. From these pressure records and the values for the elastic loads employed the tidal volume of each breath was calculated. Thus a pressure swing of

3·4 cm H_2O while breathing from the system of elastance 4·75 cm H_2O/l. represented a tidal volume of (3·4 ÷ 4·75) l. = 710 ml., while a larger pressure swing of, for example, 22·5 cm H_2O while breathing from a system of elastance 36 cm H_2O/l. represented a tidal volume of (22·5 ÷ 36) l. = 625 ml.

The lower record is the spirometer trace. The slope downward to the right represents the excess oxygen inflow over the oxygen uptake of the subject. Upon this general slope the downward deflexions represent the expulsion of air into the spirometer which occurred when the lung volume was reduced. When the lung volume increased, the spirometer record was horizontal until the excess oxygen inflow into the system of containers made up for this increase. Thereafter an escape of gas into the spirometer was again recorded.

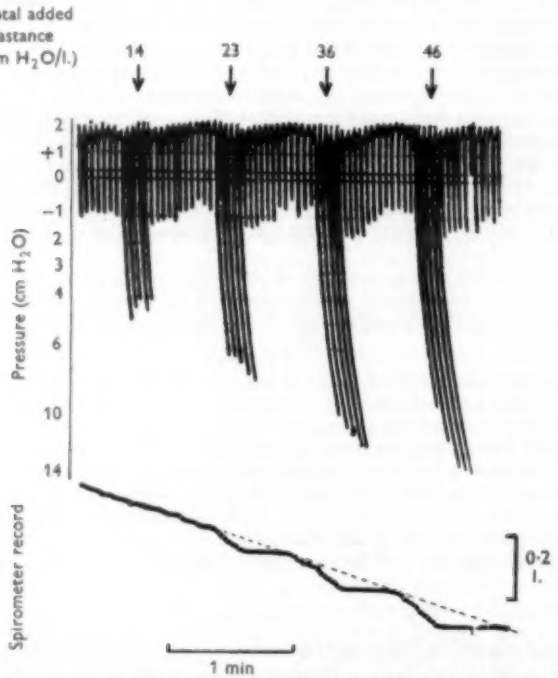

Fig. 2. Illustrative record from an anaesthetized subject (G.F.). The upper record is of pressure at the proximal end of the endotracheal tube. At the beginning the subject was rebreathing from the control load (elastance 4·75 cm H_2O/l.). Inspiration produced a downward deflexion. The application of higher loads is indicated. The lower record is the spirometer trace, sloping downwards owing to the excess O_2 inflow; volume marker 0·2 l. Note that the addition of the three highest loads caused a progressive increase in pressure swing and the expulsion of air into the spirometer. Time marker, 1 min.

From records similar to Fig. 2, tidal volumes and changes in end-expiratory level were calculated. A reconstruction of the effect upon tidal volume and end-expiratory level of an added elastic load is shown in Fig. 3. This was made from the mean values of tidal volume of each of five breaths for six separate applications of the added elastic load. The range is indicated by the vertical line through each end-inspiratory and end-expiratory point. Assuming the subject's relaxed thoracic elastance to be equal to the mean of values published (see below) the tidal volume predicted to occur if end-inspiratory muscle tension had remained constant was calculated and is represented by the dotted line. The first

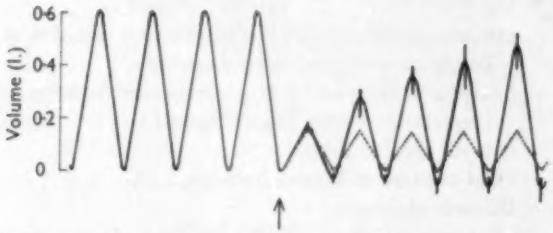

Fig. 3. Reconstructed spirogram from a conscious subject. Four control tidal volumes (inspiration upwards) followed by 5 tidal volumes from an added load of 46 cm H_2O/l. Mean of 6 experiments, the bars at the ends of the loaded breaths indicating the range of observations. The interrupted line is the predicted spirogram, assuming an average normal elastance and no change in the end-inspiratory or end-expiratory muscle tension.

breath after the application of the added elastic load was reduced in volume, and in this instance was not significantly greater than that predicted for an unchanged end-inspiratory muscle tension. However, there was an increase in tidal volume above that of the first breath with each successive breath. Further, the first expiration after the loaded inspiration is to a lower lung volume and this fall in end-expiratory level is also progressive with successive breaths.

Increased end-inspiratory muscle tension

In three conscious and three anaesthetized subjects, the additional elastic loads were introduced in a factorial manner. In one of the anaesthetized subjects the results were unsatisfactory because the level of anaesthesia varied and the experiment was interrupted by bouts of coughing. The results for the other two anaesthetized subjects and the three conscious subjects are summarized in Table 2.

A graphical representation of some aspects of these results is shown in Fig. 4. The ordinate is the ratio of the loaded tidal volume to that of the

control period and will be referred to as the tidal volume ratio. The abscissa represents the added elastic load, and starts at 4·75 cm H_2O/l., the elastance value of the control system of containers. Values for tidal volume ratios are recorded for each of the four elastances for the first and the fifth breaths only. The purpose of this figure is to compare the measured tidal volumes with those predicted, assuming end-inspiratory muscle tension to remain constant. The value of this predicted tidal volume depends upon the relaxed thoracic elastance and the elastance of the added loads.

$$P_{Ic} = V_{Tc}.El_T + V_{Tc}.El_C = V_{Tc}\,(El_T + El_C),$$

and $$P_{IL} = V_{TL}.El_T + V_{TL}.El_L = V_{TL}\,(El_T + El_L);$$

where P_{Ic} = pressure developed by the inspiratory muscles at the end of inspiration during control period,

P_{IL} = pressure developed by the inspiratory muscles at the end of inspiration during loaded breaths,

V_{Tc} = control tidal volume,

V_{TL} = tidal volume of loaded breaths,

El_T = thoracic elastance,

El_C = elastance of large container used during control period (4·75 cm H_2O/l.), and

El_L = elastance of smaller container used to produce added load.

Now, if the tension developed by the inspiratory muscles at the end of inspiration were constant,

P_{IL} would equal P_{Ic}, and

$$\frac{V_{TL}}{V_{Tc}} = \frac{El_T + El_C}{El_T + El_L}.$$ (In these experiments $V_{Tc} = V_{T_{4\cdot75}}$.)

In the case of the two anaesthetized subjects the measured values of thoracic elastance were substituted and the tidal volume ratios are shown as the two curves joining the appropriate symbols. As the attempts to measure the thoracic elastance of the conscious subjects were unsatisfactory, a range of values taken from the literature was used and provided the stippled band with limits of 7·8 and 33 cm H_2O/l. elastance. These are ± 2 S.D. of measurements of 90 anaesthetized paralysed subjects accumulated from the data of Nims, Conner & Comroe (1955), Butler & Smith (1957), Howell & Peckett (1957) and Campbell, Nunn & Peckett (1958). These subjects, in addition to being paralysed, were also supine and their average age was greater than our conscious subjects. All these factors would tend to make the assumed elastance values higher than those of our subjects. The stippled band in Fig. 4 is therefore probably higher than the best prediction would be.

The mean tidal volume ratios for each subject at each level of added

load on the first and fifth breaths are shown joined by a vertical line. To avoid superimposition of points, some have been displaced from the actual added elastance value. To maintain their relationship to the predicted tidal volume ratios, they were displaced along a line corresponding to predicted tidal volume ratio curve rather than horizontally.

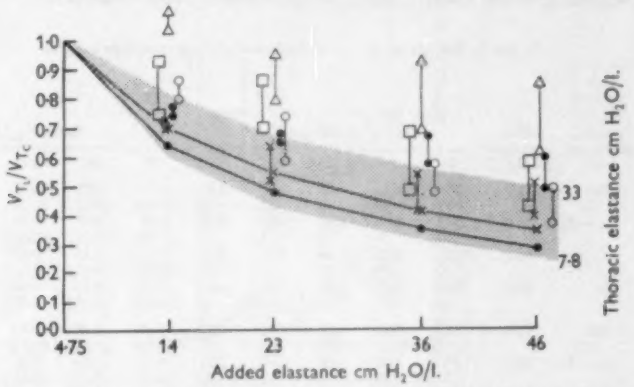

Fig. 4. Observed changes in tidal volumes compared with predicted changes, in five subjects at 4 levels of added load (from data in Table 2). $V_{T_L}:V_{T_C}$ is the ratio of the loaded to the control tidal volumes, the control being the tidal volume with an added elastance of 4·75 cm H_2O/l. Each subject is represented by a characteristic symbol (see Table 2). The vertical lines join the average ratios for the first (lower point) and fifth (upper point) loaded breath. The stippled band indicates the expected range of ratios assuming constant end-inspiratory muscle tension in the conscious subjects. The sloping lines —●— and —X— indicate the expected ratios for the two anaesthetized subjects. To avoid crowding, some of the points have been displaced obliquely (see text).

The observations which this figure illustrates are: (i) the fifth breath was always of larger tidal volume than the first, and (ii) although the tidal volume ratios of the first breath were, in most instances, within the range representing no increase in end-inspiratory muscle tension, they tend to lie in the upper part of the stippled area, suggesting that some increase in tension had probably occurred. In one of the anaesthetized subjects both the first and fifth breath ratios are above his predicted line; in the other the first breath ratios are close to the predicted line, but the fifth are well above it.

In general, the addition of an inspiratory load caused a greater reduction in tidal volume in the anaesthetized than in the conscious subjects, and the rate of increase over the five breaths was also reduced. This latter observation is represented in more detail in Fig. 5, in which the tidal volumes of the second to the fifth loaded breaths are expressed as ratios of the tidal

volume of the first breath, at two levels of added load. This figure illustrates that the proportionate increase in tidal volume over the five breaths is greater when the added load is greater, and also that the rate of increase of tidal volume over the five breaths is greater in the conscious than in the anaesthetized subject. Further studies show that this difference persists up to periods greater than 3 min. (unpublished observations).

TABLE 2. Results in two anaesthetized and three conscious subjects

Subject	Control tidal volume (V_{To} ml.) (added elastance 4·75)	Added elastance (cm H_2O/l.)	Tidal volume (V_{Tb}) after adding load of breath number					Ratio V_{Tb}: V_{To}				
			1	2	3	4	5	1/C	2/C	3/C	4/C	5/C
G.F., *M*	590	14·0	435	465	445	460	455	0·74	0·79	0·75	0·78	0·77
Age 29	(5)		(12)	(8)	(7)	(13)	(16)	(0·02)	(0·01)	(0·01)	(0·02)	(0·02)
Ht. 1·85 m	n = 22	23·0	385	390	425	425	395	0·65	0·66	0·72	0·72	0·67
S.A. 2·14 m²			(10)	(7)	(11)	(11)	(27)	(0·02)	(0·01)	(0·02)	(0·02)	(0·05)
Anaesthetized		36·0	335	355	365	365	390	0·57	0·60	0·62	0·62	0·66
elastance 11·2			(7)	(6)	(7)	(12)	(11)	(0·01)	(0·01)	(0·01)	(0·02)	(0·02)
(Symbol = ●)		46·0	280	290	320	330	350	0·48	0·49	0·54	0·56	0·59
			(9)	(7)	(8)	(14)	(9)	(0·01)	(0·01)	(0·01)	(0·02)	(0·01)
J.W., *M*	860	14·0	585	565	555	580	615	0·68	0·65	0·64	0·67	0·71
Age 46	(17)		(75)	(100)	(60)	(68)	(93)	(0·05)	(0·06)	(0·04)	(0·04)	(0·05)
Ht. 1·88 m	n = 24	23·0	445	465	495	495	540	0·51	0·54	0·57	0·57	0·63
S.A. 2·34 m²			(34)	(34)	(70)	(83)	(86)	(0·03)	(0·03)	(0·05)	(0·06)	(0·04)
Anaesthetized		36·0	350	370	420	440	465	0·40	0·43	0·49	0·51	0·54
elastance 16·9			(60)	(40)	(50)	(40)	(30)	(0·06)	(0·04)	(0·04)	(0·03)	(0·03)
(Symbol = X)		46·0	340	330	390	415	425	0·39	0·38	0·45	0·47	0·50
			(20)	(85)	(30)	(35)	(40)	(0·02)	(0·09)	(0·03)	(0·05)	(0·03)
J.F., *M*	810	14·0	660	670	705	735	720	0·82	0·82	0·87	0·91	0·88
Age 25	(10)		(30)	(20)	(25)	(30)	(30)	(0·04)	(0·03)	(0·03)	(0·03)	(0·04)
Ht. 1·70 m	n = 32	23·0	475	545	560	565	590	0·59	0·67	0·69	0·70	0·73
S.A. 1·79 m²			(60)	(55)	(65)	(50)	(35)	(0·04)	(0·03)	(0·03)	(0·03)	(0·02)
Conscious		36·0	390	415	430	450	470	0·48	0·51	0·53	0·56	0·58
			(60)	(40)	(40)	(40)	(60)	(0·04)	(0·03)	(0·03)	(0·03)	(0·04)
(Symbol = 0)		46·0	295	355	390	400	400	0·37	0·43	0·48	0·50	0·50
			(15)	(10)	(15)	(15)	(20)	(0·03)	(0·04)	(0·03)	(0·02)	(0·03)
M.J., *M*	810	14·0	575	660	680	735	730	0·71	0·82	0·84	0·91	0·90
Age 22	(30)		(50)	(45)	(50)	(40)	(30)	(0·07)	(0·06)	(0·06)	(0·05)	(0·04)
Ht. 1·90 m	n = 23	23·0	550	635	630	680	680	0·68	0·78	0·78	0·84	0·84
S.A. 2·20 m²			(30)	(30)	(45)	(60)	(45)	(0·04)	(0·04)	(0·06)	(0·07)	(0·06)
Conscious		36·0	370	465	515	515	540	0·46	0·58	0·64	0·63	0·67
			(25)	(20)	(35)	(15)	(25)	(0·06)	(0·04)	(0·05)	(0·03)	(0·04)
(Symbol = □)		46·0	330	425	405	425	455	0·41	0·52	0·50	0·52	0·56
			(25)	(30)	(15)	(15)	(15)	(0·06)	(0·06)	(0·03)	(0·03)	(0·03)
H.S., *M*	710	14·0	745	760	730	745	790	1·03	1·06	1·02	1·05	1·10
Age 33	(6)		(30)	(35)	(30)	(20)	(30)	(0·03)	(0·04)	(0·03)	(0·02)	(0·02)
Ht. 1·75 m	n = 25	23·0	565	675	680	685	675	0·79	0·94	0·95	0·95	0·94
S.A. 1·89 m²			(30)	(20)	(15)	(20)	(20)	(0·04)	(0·02)	(0·01)	(0·02)	(0·02)
Conscious		36·0	485	585	625	650	650	0·68	0·81	0·87	0·91	0·91
			(20)	(35)	(35)	(30)	(35)	(0·03)	(0·04)	(0·04)	(0·03)	(0·04)
(Symbol = △)		46·0	435	505	560	580	605	0·61	0·70	0·78	0·81	0·84
			(20)	(30)	(30)	(20)	(15)	(0·03)	(0·04)	(0·04)	(0·02)	(0·02)

Numbers in brackets are the S.E. of the mean value immediately above. In subject J.F the number of measurements of tidal volume of each breath number following the addition of the load was 8; in all other subjects, it was 6. The control tidal volumes were measured on breaths throughout the experiments, the number being indicated for each subject.

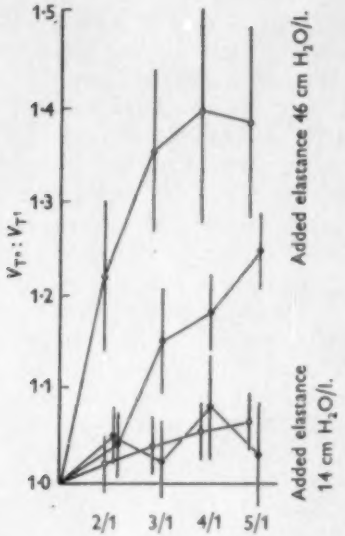

Fig. 5. Progressive increase in tidal volume after loading. $V_{T^n}:V_{T^1}$ is the ratio of the tidal volume of the second (third, fourth, fifth) loaded breath to the tidal volume of the first loaded breath. ○, conscious subject; ●, anaesthetized subject. Mean values of 8 determinations in the conscious and 6 in the anaesthetized subject; vertical bars indicate ±1 S.E. of mean. Only the findings with lowest and highest loads are shown.

Fall in end-expiratory level

An expulsion of air into the spirometer at the end of the first loaded breath (Fig. 2) occurred in all subjects. This represents a fall in the end-expiratory level (Fig. 3). This fall in level was greater when the larger elastances were employed and during the first and second breaths. The fall seemed to be greater in the conscious than in the anaesthetized subjects, but our data are insufficient to be confident. In no subject was the fall in end-expiratory level greater than 200 ml.

DISCUSSION

The most conspicuous feature of the response to inspiratory loading was the progressive development of increased tension by the inspiratory muscles which is inferred from changes in the volume of the breaths immediately following the addition of a load. A number of possibilities must be considered when seeking to explain this observation. First, it might be a simple mechanical consequence of the loading, or, secondly, it might be

due to the properties of muscle which is known to develop greater tension when shortening is impeded. Both these possibilities are excluded by the progressive nature of the response, which implies a mechanism which changes from breath to breath. Neither the mechanical conditions of the experiment, nor the properties of muscle change in this way. The increase of tension developing in this manner must represent increased activity of the α neurones supplying the inspiratory muscles. There are several possible causes for this. The first is that the underventilation produced by the added loading leads to increased chemical stimulation of the medullary or peripheral chemoreceptors with resultant increased stimulation of the spinal neurones. However, the CO_2 and O_2 tensions in the blood perfusing the main or peripheral chemoreceptors would be unlikely to be affected by the reduced ventilation within the period occupied by five breaths. We further excluded this possibility by measuring the tidal volumes of two of the conscious subjects who were subjected to a greater reduction in alveolar ventilation produced without mechanical loading. This was achieved by suddenly interposing a large dead space (1300 ml.) between the mouth-piece and valves of a closed-circuit spirometer system. In neither subject was there a detectable increase in tidal volume, nor, therefore, in inspiratory muscle tension, until the sixth breath after adding the dead space. Increases comparable with those seen after elastic loading did not occur until the tenth to twelfth breath.

The response to loading is more in keeping with an entirely nervous reflex than any of the above possibilities. The most obvious reflex mechanism to consider is one arising from stretch or similar receptors in the lungs, whose afferents pass in the vagi. Superficially, an explanation based on such a mechanism appears plausible. The limitation of inspiration by the added load would decrease the distension of the lungs and reduce the afferent discharge from stretch receptors, thus allowing greater activity of the inspiratory centre before inhibition occurs. There are three reasons for questioning this interpretation. First, such a simple mechanism would not require five breaths to develop its response. There may be some resetting of the relevant sensory endings in the lungs as the end-expiratory volume is reduced. There is no evidence in the literature of such behaviour and if it were invoked to explain our findings it would also imply that increasing end-expiratory lung volume should reduce end-inspiratory lung volume. This does not occur. Secondly, there was no change in the respiratory frequency when inspiration was loaded in a similar manner in anaesthetized subjects. Such a mechanism would almost certainly cause a prolongation of inspiration and slowing of the breathing, as well as increased force of inspiration. Thirdly, a similar progressive increase in tidal volume occurs in the rabbit, in response to elastic loading, and

persists after vagotomy. (These findings will be described in a later paper.)

The above evidence argues against the response being mediated by changes in the activity of pulmonary stretch receptors. Conditions at other intrathoracic structures are also affected by the added loads, and reflexes might arise, for example, from sites in the circulation whose pressures may alter. Such possibilities appear unlikely, particularly if similar responses occur after vagotomy.

The increased tidal volume observed in the rabbit in response to inspiratory obstruction after vagotomy, referred to above, suggests that there are reflexes arising in thoracic cage structures capable of subserving the response to inspiratory loading observed in man. The receptors and pathways involved have not been identified, but the progressive breath by breath development of the response to inspiratory loading described in this paper suggests that the reflex pathway is a complex one, and may involve higher levels of the neuraxis. Whether or not it involves the 'respiratory centre' does not seem to us a question worth considering. The central control of breathing is now known to involve so many parts of the brain, particularly the reticular formation (Liljestrand, 1958) that the concept of a 'centre' may be outmoded. The results, in fact, suggest that experiments designed to study the 'sensitivity of the respiratory centre' to various stimuli must be critically re-assessed. During the period from the first breath after loading until a new steady state would have been reached, the ventilation, work of breathing and metabolism of the respiratory muscles must all have been changing in our subjects in a manner that bore little relationship to the chemical state of the blood. It therefore becomes questionable if even steady state conditions can be used with confidence to examine the 'sensitivity' of any chemoreceptors. The ventilatory response to such stimuli may depend upon alterations at other sites, including those responsible for the effects we are studying. It is difficult to evade the impression that the respiratory stimulus or signal rhythmically generated in some part of the brain is, in effect, a demand for a certain tidal volume, and that we are studying the speed and completeness of response of a stabilizing mechanism. Teleologically such a mechanism of ventilatory control would seem much more desirable than that conventionally envisaged, in that the changes in the chemical state of the blood and a resetting of the chemoreceptors would not be required when ventilation is temporarily interfered with by postural changes, speech, eating, etc.

A second feature of the results requiring discussion is the reduction in end-expiratory lung volume during loading. This may be due to changes in thoracic blood volume (see below) or to changes in muscle tension which could either be due to more complete relaxation of the inspiratory muscles,

contraction of expiratory muscles, or both. Active expiration is improbable for the following reasons. First, the fall in volume did not exceed 200 ml. This degree of change commonly occurs when anaesthetized subjects are paralysed (J. B. L. Howell and B. W. Peckett, unpublished). This observation suggests a persistence of tone in the inspiratory muscles at the end of expiration, which, if removed, could account for the fall in end-expiratory lung volume observed in the present experiments. Secondly, recordings of intragastric pressure and the electromyogram of the abdominal muscles in one of the subjects did not provide evidence of active expiratory efforts. Active expiration cannot be completely excluded by such observations, because the techniques may not be sufficiently sensitive to detect small amounts of expiratory muscular activity.

Possible influence of changes in thoracic blood volume

Inspiration from a closed chamber resembles 'negative pressure' breathing in that the reduced intrathoracic pressure would tend to draw blood into the thorax. Conclusions about the changes in thoracic volume based on spirometry, which only indicates gas volume, may therefore be in error. The effect of this factor on our interpretation would depend on the pattern of variation of intrathoracic blood volume. Several patterns of respiratory variation in intrathoracic blood volume can be envisaged as following the addition of an elastic load. None of them can explain our results and most of them would operate in such a way as to cause the calculated changes in thoracic volume to be underestimates.

To exclude the possibility that the progressive increase in tidal volume is due to a varying shift of blood we made two further observations. First, we recorded abdominal and thoracic stethograms, using distensible tubing strapped round the trunk. These showed a progressive increase during elastic loading. Secondly, we recorded intrathoracic pressure changes by means of an oesophageal balloon. This also showed a progressive increase in respiratory variation during elastic loading. These observations dispose of the possibility that the end-inspiratory muscle tension is constant and the increasing tidal (gas) volume is due to a diminishing tidal (blood) volume.

SUMMARY

1. The breathing of nine conscious and three anaesthetized human subjects was elastically loaded by making them rebreathe from closed rigid drums.

2. The effects of loads of up to 46 cm H_2O/l. were studied for five breaths. Tidal volume and changes in end-expiratory lung volume were measured. Changes in the tension developed by the inspiratory

muscles at the end of inspiration were inferred from the changes in lung volume and the thoracic elastance.

3. There was a progressive increase in the tidal volume and, by inference, in end-inspiratory muscle tension from the first to the fifth loaded breath. This effect was greater with larger loads and was greater in the conscious subjects.

4. The end-inspiratory muscle tension was probably increased in the first loaded breath in most subjects.

5. The end-expiratory lung volume fell during the loading, particularly with the higher loads, but the fall did not exceed 200 ml.

6. Explanations for the findings are examined, including a simple mechanical effect, the properties of muscle, changes in thoracic blood volume, reflex action. The findings are considered to be due to reflex action and evidence is presented that this is a somatic reflex not necessarily invclving afferents from the lungs.

7. A mechanism with the speed of response we have found would tend to stabilize ventilation more efficiently than those usually envisaged.

We are grateful to Mr Rainsford Mowlem for allowing us to study patients under his care, and to the subjects for their co-operation. We thank Dr O. W. Hill for critical suggestions.

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ADENOSINETRIPHOSPHATASE ACTIVITY AND THE ACTIVE MOVEMENTS OF ALKALI METAL IONS

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It is now generally accepted that the mammalian red cell, like nerve and probably like most cells, maintains its ionic composition by pumping potassium inwards and sodium outwards. In red cells there is good evidence that the energy for this pumping comes from glycolysis (Danowski, 1941; Harris, 1941; Maizels, 1949). It is also well known from the work of Clarkson & Maizels (1952), Caffrey, Tremblay, Gabrio & Huennekens (1956), and Herbert (1956), that the red cell membrane possesses adenosinetriphosphatase (ATP-ase) activity. The experiments to be described in the present paper were designed to see whether this ATP-ase activity is connected with the mechanisms responsible for ion transport. A preliminary account of these experiments has already been published (Dunham & Glynn, 1960).

Four features characteristic of the transport system are (Glynn, 1957*a*):

1. The active movements of sodium and potassium are linked, in the sense that the cell is unable to pump sodium out unless potassium is available outside to be pumped in (Harris & Maizels, 1951; Shaw, 1954; Glynn, 1956).

2. Active transport is prevented by cardiac glycosides at concentrations of about 10^{-5} g/ml. (Schatzmann, 1953; Kahn & Acheson, 1955; Glynn, 1957*b*).

3. Modified cardiac glycosides in which the lactone ring is saturated or attached with the α configuration at C_{17} are much less active, or inactive (Glynn, 1957*b*).

4. At much lower concentrations of glycoside, inhibition can be prevented by raising the potassium concentration in the fluid *outside* the cells (Glynn, 1957*b*).

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Washed human red cell ghosts have been incubated in salt solutions containing ATP, and the rate of splitting has been followed by measurement of the inorganic phosphate formed. The response of the ATP-ase to the factors known to affect cation transport has been observed.

METHODS

Preparation of ghosts

First method. Freshly drawn human blood was used in all experiments. Clotting was prevented with heparin ('Pularin', Evans Medical Supplies) in a concentration of about 5000 u./100 ml. blood. The blood was centrifuged and the plasma and buffy coat removed by suction; the cells were then washed three times with isotonic saline by alternate resuspension and spinning. White cells were almost completely eliminated by removal of the top-most layer of cells with the supernatant after each wash.

The cells were lysed by adding them quickly to 10 volumes of stirred ice-cold glass-distilled water containing 0·1 mM cysteine. The haemolysate was allowed to stand for 10 min, and the cells were then spun down (6 min at 16,000 *g*) and washed with about 6 volumes of ice-cold glass-distilled water of which the pH had been adjusted to between 7·0 and 7·2 and to which a trace of cysteine had been added. The washing was repeated two or three times, until the supernatant contained only a trace of pink colour although the cells remained definitely pink. Some salt was added at this stage (to give a concentration between 1/3 isotonic and isotonic) and the suspension was pipetted into the graduated tubes in which incubation was to be carried out. The quantity of cells added to each tube was generally that which gave a haematocrit of about 10 % during incubation (calculated on the original volume of the cells, not the volume of the ghosts). The addition of salt before pipetting was found to be necessary, since otherwise the ghosts tended to clump, and reproducible results could not be obtained.

Second method. Ghosts prepared by the method just described were usually satisfactory, but occasionally the activity was two or three times as great as was expected and there was very little inhibition by cardiac glycosides. As will be shown later, this is what would be expected if the preparation had been contaminated with calcium ions and, though calcium contamination was never demonstrated, it was found that the properties of a supposedly contaminated preparation returned towards normal if the cells were washed with an isotonic solution of magnesium chloride of a 1 mM solution of ethylenediaminetetraacetate.

The procedure was therefore modified in that isotonic $MgCl_2$ was used to wash the cells before lysis, and after lysis strong $MgCl_2$ solution was added to make the concentration isotonic again. The ghosts were then washed in the usual way with chilled neutral glass-distilled water containing a trace of cysteine.

Third method. Ghosts prepared by the second method always had a normal fraction of glycoside-sensitive activity, but the absolute amount of activity was usually rather low and occasionally very low—about 1/5 of normal. It was thought that the restoration of isotonicity might have made many of the ghosts impermeable to ATP (cf. Székely, Mányai & Straub, 1952), and in one preparation whose activity was very low only 1/5 of the total volume of the ghosts was found to be accessible to ATP. Freezing and thawing increased the activity to normal—presumably by making the membranes leaky again—and in the third method the ghosts were frozen and thawed twice, after washing but before the addition of salt.

Reagents

ATP was obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio, and was purified on Dowex 1 ion-exchange columns by the following procedure: The ATP disodium salt was dissolved in a small quantity of water, and sufficient NaOH added to

bring the pH to between 6 and 8. This solution was poured onto 4 cm (1·5 ml.) columns of Dowex 1 resin, allowing about 0·25 g ATP per column. Each column was then washed with about 50 ml. of a solution containing 20 m-mole HCl and 20 m-mole NH_4Cl per litre. This solution brought down traces of adenosinemonophosphoric acid and adenosinediphosphoric acid. After the ammonium ions had been got rid of by washing each column with about 20 ml. of distilled water, the ATP was eluted with about 10 ml of chilled 250 mM-HCl. The effluent was received in chilled tubes and immediately neutralized with an ice-cold solution of trishydroxymethylaminomethane (Tris) hydroxide. The concentration of ATP was determined by U.V. absorption measurements at 259 mμ. After use the resin columns were washed with about 20 ml of 1 M-HCl and then with water until the pH of the effluent was about 5.

Salts. Except where otherwise stated, the salts used were of 'analytical reagent grade'. The 'spectroscopically pure' salts used in some of the experiments were supplied by Johnson Matthey and Co. Ltd.

Cardiac glycosides. Strophanthin was supplied by British Drug Houses Ltd. Scillaren A and hexahydroscillaren A were a gift from Messrs Sandoz of Basle. Cymarin and 17α-cymarin were a gift from Professor Reichstein of Basle. The glycosides were dissolved in 80 % ethanol (AR grade), and the alcoholic solutions were diluted with water.

Tris was the 'Tris 121 grade' supplied by the Sigma Chemical Company, St Louis, Missouri.

Cysteine was supplied by the Nutritional Biochemical Corporation.

All reagents were made up in glass-distilled water.

Procedure

Incubation was carried out in 5 or 10 ml. graduated tubes with glass stoppers. The reaction was generally started by the addition of ATP in a volume of fluid equal to one tenth of the total volume, so that the cooling effect was small. At the end of the incubation period the tubes were cooled in iced water for 1 min, and then ice-cold 55 % trichloroacetic acid was added to make the final concentration 5 %. The tubes were kept in iced water until it was convenient to spin down the precipitated protein and estimate the inorganic phosphate in a portion of the supernatant by the method of Fiske & Subbarow (1925). The cooling before the addition of trichloroacetic acid was found to be necessary to get complete deproteinization; if it was omitted the supernatants looked clear after spinning but became cloudy on addition of the strongly acid phosphomolybdate. The need to cool could probably have been avoided by the use of much larger volumes of more dilute trichloroacetic acid, but this would have produced a much larger final volume and so would have decreased the sensitivity of the method. The time during which each tube was kept in iced water before analysis was noted, and a correction was made for the small non-enzymic breakdown of ATP during this period and during the colour development. A small blank correction was also made, to allow for the trace of colour developed in supernatants from control tubes to which no ATP had been added. Preliminary trials showed that the presence of 1 mM cysteine (the concentration used in all experiments) did not affect the phosphate determinations, though concentrations of 5 mM or more caused large errors.

In experiments involving large numbers of tubes, the ATP was added serially at 15 sec intervals at the beginning of the experiment, and the tubes were removed from the water-bath to iced water at 15 sec intervals at the end. Trichloroacetic acid was added to the tubes in the same order exactly 1 min later. The large number of tubes used in some of the experiments and the necessity of washing the syringe at certain stages, to avoid contamination of one tube by the contents of another, meant that sometimes the tubes at the end of a run had been incubated at 37° C for perhaps 20 min longer than those at the beginning. Fortunately, it was found that incubation in the absence of ATP for 1 hr at 37° C led to a decrease in enzyme activity of just under 5 %, so the change in activity between the beginning and end of a run could be neglected.

RESULTS

Preliminary experiments (Figs. 1 and 2) showed that, under the conditions chosen, the amount of phosphate formed in a given time was proportional to the quantity of cells present; for a given quantity of cells the amount increased linearly with time for 1½ hr. In one or two experiments there was a falling off in activity of between 5 and 10% during

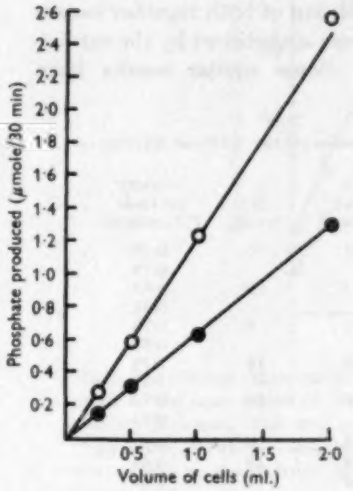


Fig. 1

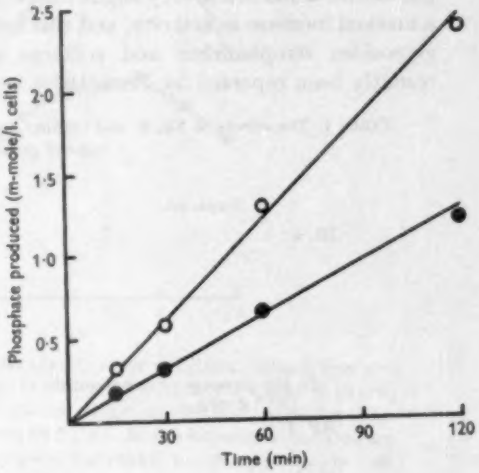


Fig. 2

Fig. 1. The effect of enzyme concentration on the rate of liberation of phosphate from ATP by red cell ghost ATP-ase. Ghosts were prepared by the first method. Temp. 37·1° C; pH 7·1; ATP 2 mM; Mg 5 mM; Na 132 mM; K 10 mM; cysteine 1 mM; ethylenediaminetetraacetate 1 mM; Tris 28 mM. Duration 30 min. ○ glycoside absent; ● strophanthin 10^{-6} g/ml.

Fig. 2. Time curve for the liberation of phosphate from ATP by red cell ghosts in the presence and absence of strophanthin. This experiment was done on the same preparation and under the same conditions as that shown in Fig. 1: symbols as in Fig. 1.

the first hour; the reason for this was never discovered. In subsequent experiments the amount of phosphate formed by a given quantity of cells in a fixed time, usually ½ hr or 1 hr, was used as a measure of activity.

The effects of Na, K and cardiac glycosides

If part of the ATP-ase activity is connected with the pump, and the pump only pumps if both sodium and potassium are present, one would

expect to find this part of the activity dependent on the presence of both sodium and potassium. One might also expect that any activity which appeared only in the presence of both sodium and potassium would disappear on the addition of cardiac glycosides at concentrations known to inhibit the pump. These expectations are realized in the results of two experiments shown in Table 1.

It is clear that a large part of the ATP-ase activity occurs in the absence of alkali metal ions. Addition of sodium alone has no effect; addition of potassium alone has a very slight effect; addition of both together causes a marked increase in activity, and this increase is abolished by the cardiac glycosides strophanthin and scillaren A. Some similar results have recently been reported by Post (1959).

TABLE 1. The effects of Na, K and cardiac glycosides on the ATP-ase activity of red cell ghosts

Expt. no.	[Na] (mM)	[K] (mM)	Activity (m-mole P/l. cells/hr)
IG. 4	0	0	0·75
			0·78
	0	16	0·82
			0·84
	16	0	0·73
			0·83
	16	16	1·34
			1·37
In the presence of strophanthin (10^{-4} g/ml.)	16	16	0·78
			0·85
RP. 1	0	0	0·57
	0	16	0·64
	64	0	0·55
	64	16	1·33
In the presence of scillaren A ($1·7 \times 10^{-6}$ g/ml.)	64	16	0·58

Conditions of experiments. Ghosts prepared by the third method. Duration 1 hr; temp. 37° C; pH 7·0; ATP 1·5 mM; Mg 0·5 mM in Expt. IG. 4, 0·96 mM in Expt. RP. 1; cysteine 1 mM; Tris to make up 160 mM; 'Specpure' Na and K.

It is convenient, in what follows, to refer to the activity that depends on the presence of both sodium and potassium as 'glycoside-sensitive' and to the remainder as 'glycoside-insensitive'. This is not meant to imply that two separate enzymes are responsible, though they may be.

The effect of pH

Figure 3 shows the effect of pH in the presence and absence of cardiac glycosides. The solution was buffered with 35 mM Tris, but over the lower range of the curve the buffering was provided mainly by the ATP itself. The quantity of hydrogen ions liberated during the hydrolysis was calculated to be insufficient to upset the pH in all except the lowest two tubes, where a drop of $\frac{1}{2}$ in pH probably occurred during the run.

The results are not accurate enough to make it worth while to plot the difference curve, which would give the pH sensitivity of the glycoside-sensitive component, but it is obvious that the effects of pH on the two components are not very different.

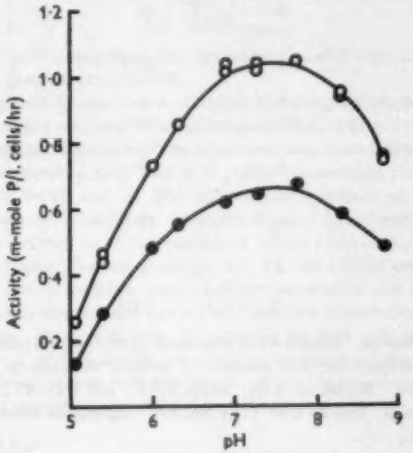


Fig. 3. The effect of pH on the ATP-ase activity of red cell ghosts. Ghosts were prepared by the second method. Temp. 37·1° C; ATP 1·5 mM; Mg 2 mM; K 10 mM; cysteine 0·75 mM; Tris 35·3 mM; Na to make up 160 mM. Duration 1 hr. ○ glycoside absent; ● + strophanthin 10^{-4} g/ml. In this experiment the reaction was started by the addition of the ghost suspension to solutions already containing ATP.

Activation by divalent cations

The ATP-ase studied by Herbert (1956) presumably corresponds to our 'glycoside-insensitive component', as potassium was not present in Herbert's experiments. Herbert's enzyme was strongly activated by magnesium ions; calcium activated weakly when present alone but reversed the activation due to magnesium.

The results given in Table 2 show that even if sodium and potassium are both present there is very little activity in the absence of magnesium. The relation between activity and magnesium concentration, both in the presence and absence of strophanthin, is shown in Fig. 4. The difference, representing the glycoside-sensitive component, is shown as a dotted line.

Since one way of explaining the activating effect of magnesium is to suppose that a complex of Mg and ATP is the true substrate, the concentration of Mg–ATP complex has been calculated for each magnesium concentration. From the titration curves of ATP between pH 8 and pH 2 in the presence and absence of magnesium, Burton & Krebs (1953) conclude that $MgATP^{2-}$ is the principle complex. Burton (1959) has measured the formation constant of this complex, and ΔH for its formation, by an ingenious method in which the

TABLE 2. Dependence of the ATP-ase activity of red cell ghosts on the presence of magnesium

Expt. no.	[Mg] (mM)	Strophanthin (g/ml.)	Activity (m-mole P/1. cells/hr)
DD. 1	0	0	−0·018
			0·007
			−0·060
	0	10^{-4}	−0·035
			0·011
			−0·011
	1·5	0	0·425
	1·5	10^{-4}	0·245
JD. 1	0	0	0·036
			0·060
			0·055
	0	10^{-4}	0·043
			0·046
			0·031
	2	0	0·490
			0·504
	2	10^{-4}	0·271
			0·281

Conditions of experiments. Ghosts were prepared by the second method and then washed three times with a buffered isotonic solution of sodium chloride to get rid of magnesium bound to fixed anions. Duration 1 hr; temp. 37° C; pH 7·1; ATP 1·5 mM; Na 120 mM; K 10 mM; cysteine 1 mM; Tris 30 mM. (The negative signs arise when the colour developed is less than the blank.)

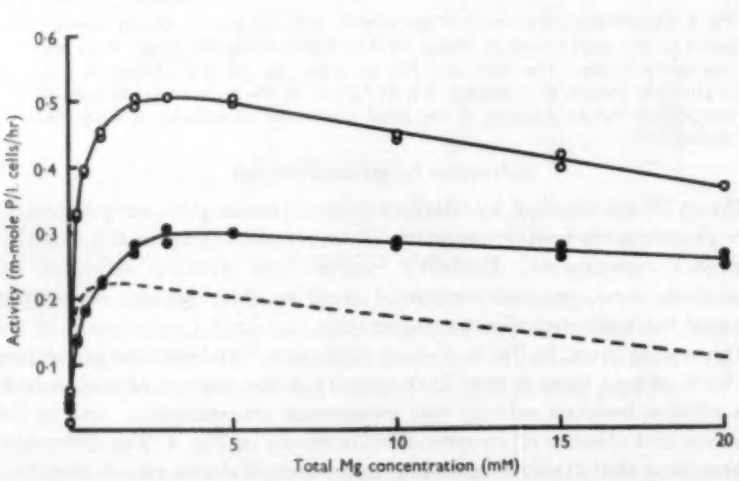


Fig. 4. The effect of Mg on the ATP-ase activity of red cell ghosts. Ghosts were prepared by the second method and then washed three times with a buffered isotonic solution of sodium chloride to get rid of Mg bound to fixed anions. Temp. 37° C; pH 7·1; ATP 1·5 mM; Na 120 mM; K 10 mM; cysteine 1 mM; Tris 30 mM. Duration 1 hr. (○) glycoside absent; (●) strophanthin 10^{-4} g/ml. The difference between the two curves is shown by the dotted line, which therefore represents the 'glycoside-sensitive' component.

concentration of free magnesium is calculated from the absorption spectrum of 8-hydroxyquinoline present in the solution. From Burton's data referring to the formation of complex at 25° C and pH 8 and in the presence of 100 mM-K, it is possible to calculate that the apparent formation constant at 37° C and pH 7 must be about 14000, where the apparent formation constant is defined by the ratio

$$\frac{[MgATP^{2-}]}{[Mg^{2+}][ATP_{\text{total free}}]}.$$

This figure has been used to calculate the concentration of complex at each concentration of magnesium in the present experiments.

The falling off in glycoside-sensitive activity at high magnesium concentrations suggests that free magnesium might compete with the complex, and, in fact, the observed relationship between glycoside-sensitive activity and the concentrations of complex and of free magnesium fit simple competitive kinetics very well if K_m (the dissociation constant of the enzyme-substrate complex) is 0·077 and K_i (the dissociation constant of the enzyme inhibitor complex) is 0·84. However, when these values for K_m and K_i are used to predict the change in glycoside-sensitive activity in an experiment in which total magnesium is kept constant and total ATP is increased (see, for example, Fig. 5), the results are wildly out. Another possibility that has been considered, suggested by the drop in the last point in Fig. 5, is that Mg-ATP complex is the substrate and that both free magnesium and free ATP act as competitive inhibitors. It should then be possible to find values for three constants, K_m, K_i and K_j (where K_i and K_j are the dissociation constants of the two enzyme-inhibitor complexes) which are compatible with the results of both experiments. In fact this has not been possible and the hypothesis must be rejected.

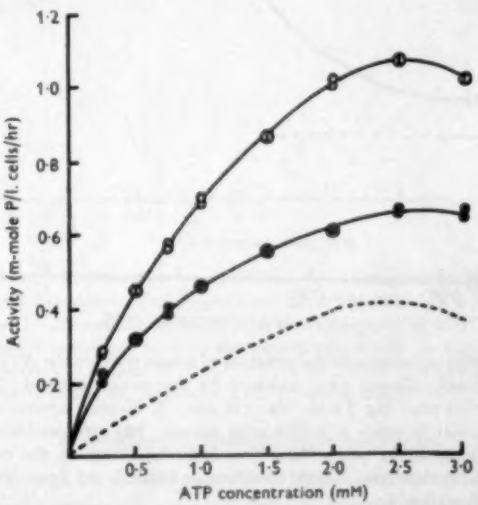


Fig. 5. The effect of ATP concentration on the ATP-ase activity of red cell ghosts. Ghosts were prepared by the second method. Temp. 37° C; pH 7·1; Mg 3 mM; Na 110 mM; K 10 mM; cysteine 1 mM; Tris 30 mM. Duration 1 hr. (○) glycoside absent; (●) strophanthin 10^{-4} g/ml. The difference between the two curves is shown by the dotted line, which therefore represents the 'glycoside-sensitive' component.

Calcium. In the presence of magnesium quite small amounts of calcium increase the ATP-ase activity two- or threefold; higher concentrations of calcium lead to inhibition (Fig. 6). The optimum level of calcium depends on the magnesium concentration (Fig. 7), being roughly three times as high when the magnesium concentration is raised by a factor of three.

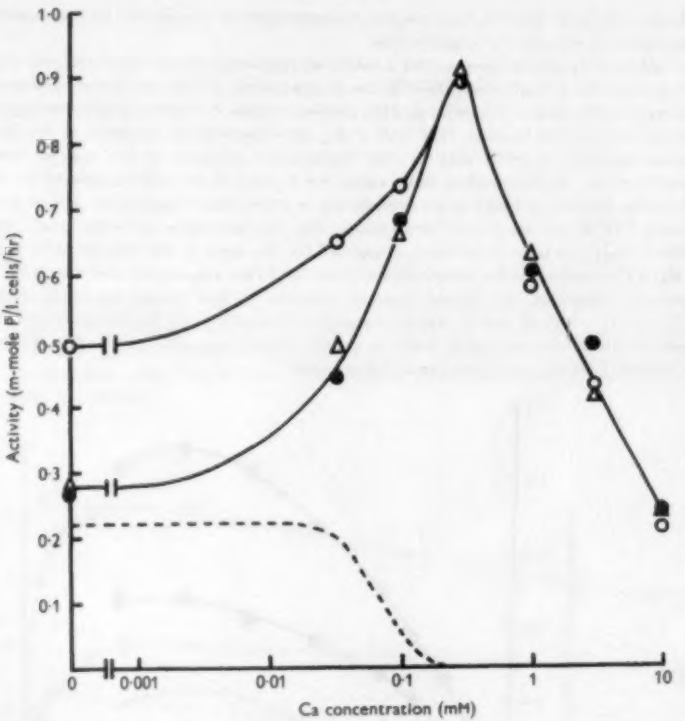


Fig. 6. The effect of calcium in the presence of magnesium on the ATP-ase activity of red cell ghosts. Ghosts were prepared by the second method. Temp. 37° C; pH 7·0; ATP 1·5 mM; Mg 2 mM; Na 110 mM; K 20 mM; cysteine 1 mM; Tris 30 mM. Duration 30 min. (○) glycoside absent; (●) strophanthin 10^{-6} g/ml.; (△) K-free, replaced by extra Tris. The difference between the two curves is shown by the dotted line, which therefore represents the 'glycoside-sensitive' component. Semi-log. scale.

This suggests that the inhibitory effect of calcium depends on a straight Mg–Ca competition. The activation by calcium affects only the glycoside-insensitive component; the glycoside-sensitive component is inhibited even at low calcium concentrations (Fig. 6). Figure 6 also shows that, as might

be expected, the absence of potassium has much the same effect as the presence of strophanthin.

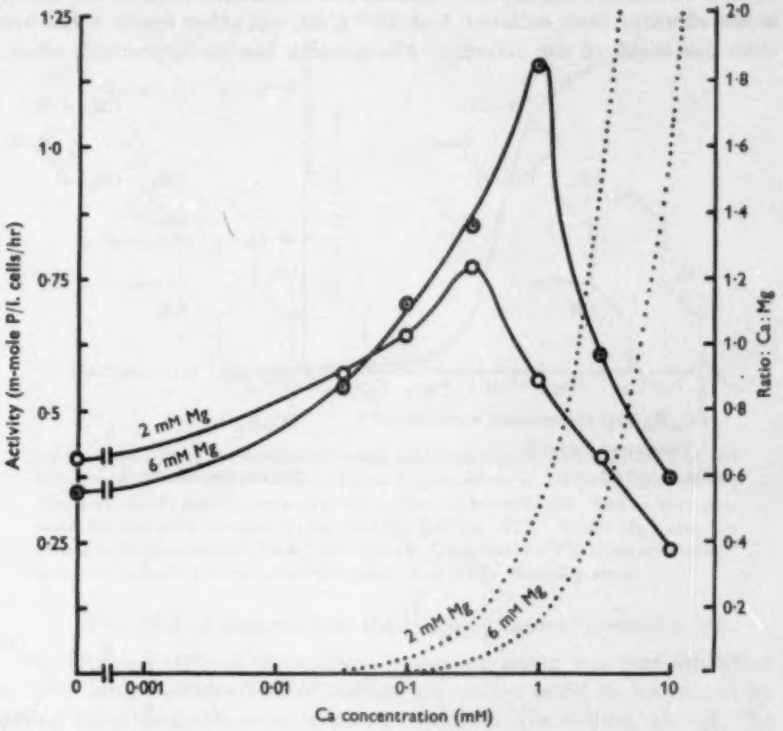


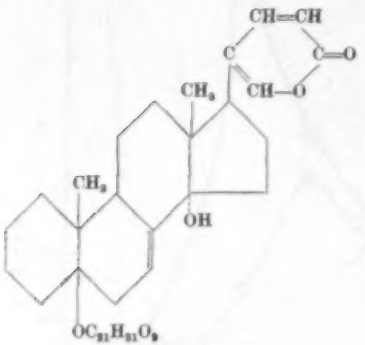
Fig. 7. The effect of calcium at two different levels of magnesium on the ATP-ase activity of red cell ghosts. The dotted lines show the ratio of Ca to Mg. Ghosts were prepared by the second method. (The ghost preparation used in this experiment is different from that used in the experiment shown in Figure 6, and has slightly less activity.) Temp. 37° C; pH 7·0; ATP 1·5 mM; Na 110 mM; K 20 mM; cysteine 1 mM; Tris 30 mM. Duration 1 hr. Semi-log. scale.

Molecular requirements for inhibition

It was mentioned in the introduction that certain modifications to the cardiac glycoside molecule greatly reduce the inhibitory effect on ion pumping. The formulae of two glycosides that are powerful inhibitors of ion pumping, and of two related compounds that are much less active in this respect, are shown on p. 284.

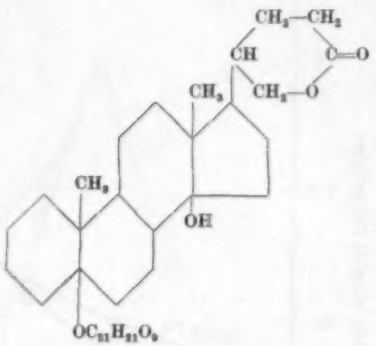
Figure 8 shows a 'dose-effect curve' for the inhibition of the glycoside-sensitive component of the ghost ATP-ase by scillaren A. Marked on the

ordinate are the effects of 10^{-6} g/ml. of cymarin, 17 α-cymarin, and hexahydroscillaren A. It is clear that cymarin and scillaren A are about equally effective. Hexahydroscillaren A at a concentration of 10^{-6} g/ml. is less effective than scillaren A at 10^{-7} g/ml.—in other words it has less than one tenth of the activity. 17α-cymarin has no appreciable effect.

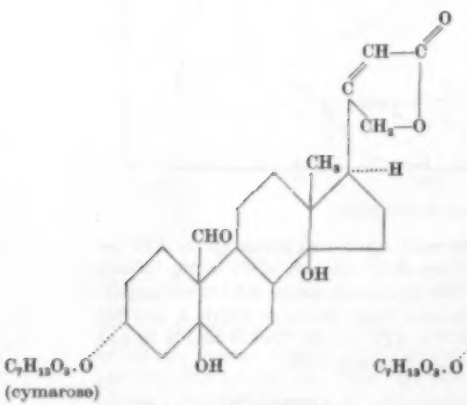


(rhamnose-glucose)

Scillaren A* (active)

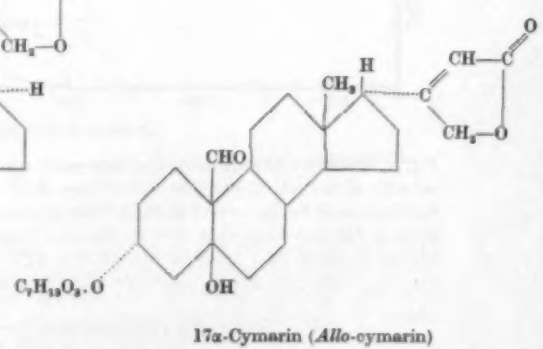


Hexahydroscillaren A



(cymarose)

Cymarin (active)



17α-Cymarin (*Allo*-cymarin)

* In the paper by Glynn (1957*b*) the formulae of scillaren A and of emicymarin are unfortunately shown with the wrong configuration at C_{17}.

So the same molecular features seem to be important for ATP-ase inhibition as are important for inhibiting the ion pump, and also, incidentally, for 'cardiotonic' action (Wollenberger, 1954). In this respect the effect of cardiac glycosides on the membrane ATP-ase differs from their effect on

the viscosity of cardiac actin solutions, for which the molecular requirements are much less stringent (Horvath, Kiraly & Szerb, 1949; Snellman & Gelotte, 1950).

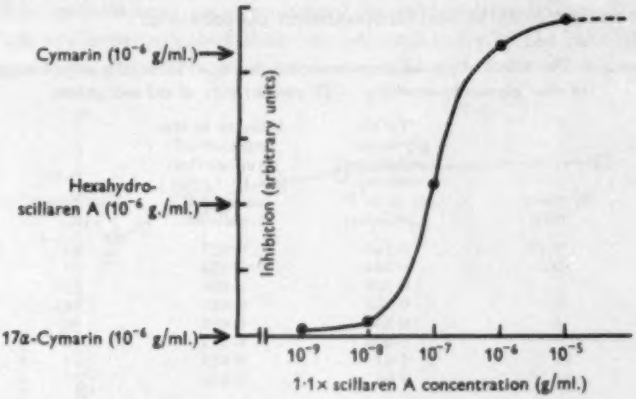


Fig. 8. Features of the cardiac glycoside molecule required for inhibition of red cell ghost ATP-ase. The continuous line is a 'dose–effect' curve for scillaren A. The effects of certain related compounds are shown on the ordinate. Ghosts were prepared by the third method. Temp. 37·1° C; pH 7·1; ATP 1·5 mM; Mg 1 mM; Na 64 mM; K 16 mM; cysteine 1 mM; 'Tris' 80 mM. Duration 1 hr. The tubes were placed in the water-bath 45 min before the addition of ATP. Semi-log. scale.

The effect of potassium on inhibition by cardiac glycosides

The fourth feature of the sodium–potassium pump was that inhibition by very low concentrations of cardiac glycosides could be prevented by raising the potassium concentration outside the cells (Glynn, 1957*b*). The effect resembled competitive inhibition, though competition between potassium ions and cardiac glycoside molecules does not seem very likely and the data did not accurately fit simple competitive kinetics. Table 3 shows the effect of potassium concentration on the inhibition of the glycoside-sensitive component of the ATP-ase by strophanthin at a concentration of 5×10^{-8} g/ml. The decrease in inhibition as the potassium concentration is raised is striking. Two experiments with slightly higher concentrations of strophanthin gave similar results, except that the reversal was less complete, and remained incomplete even when the potassium concentration was increased further. Thus, with strophanthin at a concentration of 10^{-7} g/ml. inhibition of the 'sensitive' component decreased from about 90% with 0·25 mM-K^+ to 18% with 8 mM-K^+ and then remained at about this level from 15 to 64 mM-K^+. With strophanthin at 2×10^{-7} g/ml. inhibition was 35% at 8 mM-K^+, 39% at 16 mM-K^+, and

35% at 32 mM-K^+, the differences between these figures not being significant. The interpretation of this levelling off is not clear but it cannot be explained on simple competitive kinetics. It could conceivably be due to a separate non-competitive inhibition, either by strophanthin itself or an impurity in the strophanthin preparation.

TABLE 3. The effect of potassium concentration on inhibition by strophanthin of the 'glycoside-sensitive' ATP-ase activity of red cell ghosts

K concn. (mM)	Total glycoside-sensitive activity (m-mole P/l. cells/hr)	Activity in the presence of strophanthin (5×10^{-6} g/ml.) (m-mole P/l. cells/hr)	Inhibition (%)
0·25	0·188	0·027	86
0·5	0·364	0·134	63
1	0·619	0·290	53
2	0·778	0·498	36
4	0·855	0·686	20
8	0·926	0·782	15
16	1·02	0·823	19
32	1·04	0·964	7

Conditions of experiment. Ghosts prepared by the third method. Duration 1 hr; temp. 37° C; pH 7·2; ATP 1·5 mM; Mg 0·5 mM; Na 60 mM; cysteine 1 mM; Tris to make up 160 mM. The tubes were placed in the water-bath an hour before the addition of ATP. The total glycoside-sensitive activity was determined with 10^{-4} g strophanthin/ml. and at its highest accounted for 56% of the total ATP-ase activity (see Fig. 9).

Sodium and potassium interaction

So far nothing has been said about the concentrations of sodium and potassium necessary to activate the glycoside-sensitive component of the ATP-ase. Figure 9 shows that with 60 mM-Na^+ present the activity increases with increasing potassium concentration up to about 4 mM-K^+ and then levels off. The concentration of potassium for half-maximum activity is about 1 mM. A similar result is shown by the top curve of Fig. 10. When activity is plotted as a function of sodium at a fixed potassium concentration of 16 mM (Fig. 11, top curve), activity increases up to about 32 mM-Na^+ and then levels off. The sodium concentration for half-maximum activity is about 4 mM. However, these statements do not adequately summarize the behaviour of the ATP-ase, because the effect of sodium concentration depends on the concentration of potassium and vice versa. Thus, in Fig. 11, although with 16 mM-K^+ activity increases with increasing sodium concentration till 32 mM-Na^+ and then levels off, with only 0·5 mM-K^+ there is an optimum at 8 mM-Na^+, and higher levels of sodium give less activity. Similarly, looking at the results in Fig. 11 the other way round, with 64 mM-Na^+ activity increases as potassium is raised from 0·5 to 2 to 16 mM, but with 2 mM-Na^+ there is slightly less activity at

2 mM-K^+ than at 0·5 mM-K^+, and less again at 16 mM-K^+. Again, in Fig. 10, with 64 mM-Na^+ activity is more than doubled as potassium is raised from 0·5 to 8 mM, while with only 2 mM-Na there is a slight decrease in activity over this range.

All these results may be summarized by saying that both sodium and potassium are necessary, but that for optimum activity the ratio between them must be neither too great nor too small.

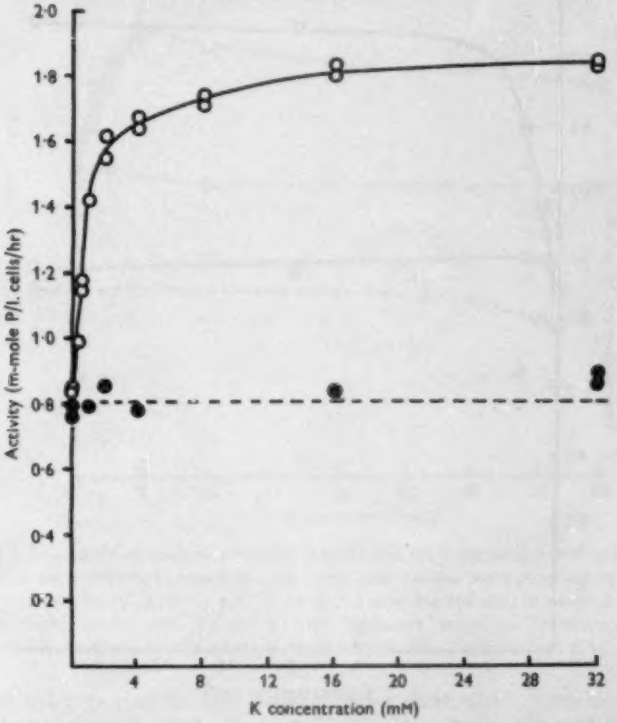


Fig. 9. The effect of potassium concentration on the ATP-ase activity of red cell ghosts in the presence and absence of strophanthin. 60 mM-Na; ○ glycoside absent; ● strophanthin 10^{-4} g/ml. The results plotted here were obtained on the same preparation, under the same conditions and at the same time as those given in Table 3.

DISCUSSION

The dependence of ion transport on glycolysis in some cells, on respiration in others, and on either in yet others, suggests that energy may be supplied to the pump in the form of ATP rather than directly from any of the individual steps in glycolysis or respiration. Support for this idea

comes from experiments with dinitrophenol. Dinitrophenol uncouples respiration from phosphorylation and is found to inhibit ion pumping in nerve and nucleated red cells, which depend on respiration, but not in mammalian red cells, which depend on glycolysis (Maizels, 1954; Hodgkin & Keynes, 1955). Further support for the role of ATP comes from the experiments of Caldwell (1956) on squid axons, and of Dunham (1957*a*, *b*)

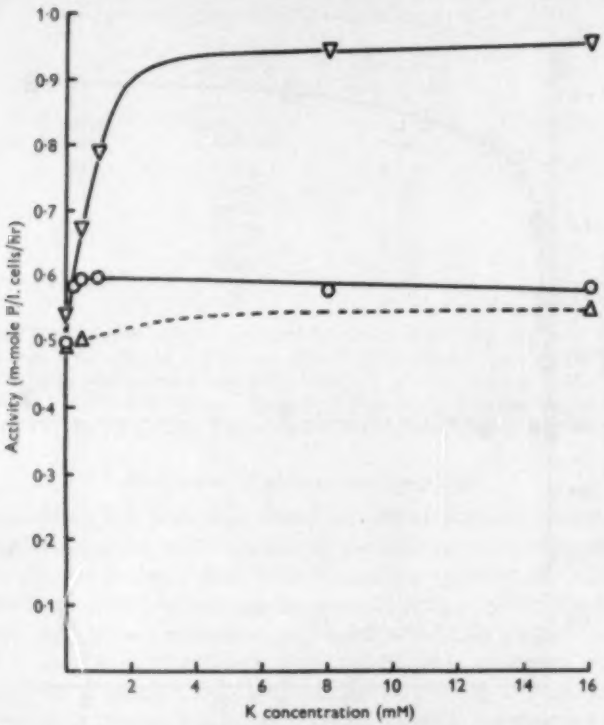


Fig. 10. The effect of potassium concentration on the ATP-ase activity of red cell ghosts at two different sodium concentrations. Ghosts were prepared by the third method. Temp. 37·1° C; pH 7·1; ATP 1·5 mM; Mg 1 mM; cysteine 1 mM; Tris to make up 160 mM. Na and K were 'Specpure' reagents. Duration 1 hr. ▽ 64 mM-Na; ○ 2 mM-Na; △ 0 mM-Na.

and Whittam (1958) on mammalian red cells, showing a correlation between the rate of ion transport and the concentration or rate of splitting of 'energy-rich' phosphate. More direct evidence is provided by experiments in which ATP was injected intracellularly into cyanide-poisoned squid axons (Caldwell & Keynes, 1957), or incorporated into 'reconstituted'

ghosts (Gardos, 1954), or removed from intact red cells by poisoning glucose-free cells with iodoacetate and then allowing them to phosphorylate added glucose (Dunham, 1957*b*).

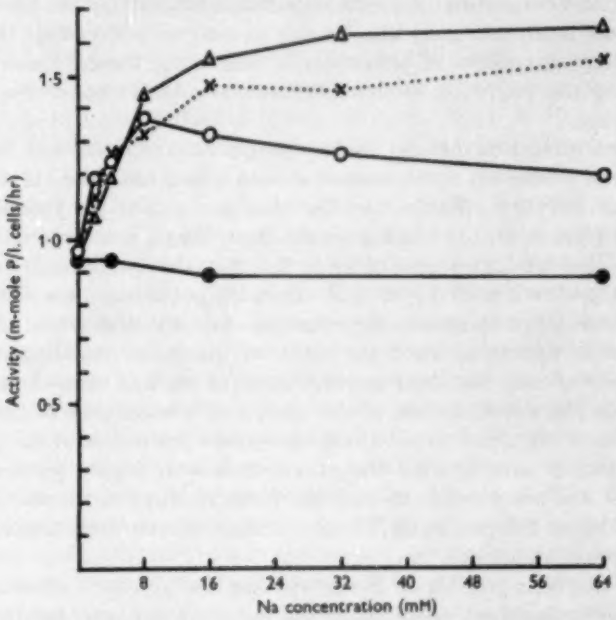


Fig. 11. The effect of sodium concentration on the ATP-ase activity of red cell ghosts at three different potassium concentrations. Ghosts were prepared by the third method. Temp. 37·2° C; pH 7·2; ATP 1·5 mM; Mg 0·5 mM; cysteine 1 mM; Tris to make up 160 mM. Na and K were 'Specpure' reagents. Duration 1 hr. △ 16 mM-K, × 2 mM-K, ○ 0·5 mM-K, ● 16 mM-K + strophanthin 10^{-4} g/ml.

The results reported in the present paper show that there is a close parallelism between the properties of an ATP-ase in the red cell membrane, and the properties of the pump responsible for shifting ions across that membrane. It is difficult to avoid the conclusion that the action of this ATP-ase somehow provides energy for the transport of sodium and potassium.

If, as seems likely, the sodium and potassium ions that activate the ATP-ase are also the ions that are pumped across the membrane, then it follows that *external* potassium and *internal* sodium are the activating ions. As the ghosts used in these experiments were highly permeable to sodium and potassium, there is no direct evidence on this point, but there is some

indirect evidence relating to potassium ions. The prevention by potassium of glycoside inhibition in intact cells is due to *external* potassium, because, in the relevant experiments, the internal potassium was high and remained unchanged. Unless the resemblance between this effect and the potassium reversal of ATP-ase inhibition is coincidental, it follows that the prevention of ATP-ase inhibition must also be due to *external* potassium. It is not certain that the effects of potassium in activating the ATP-ase and in preventing the inhibition of the ATP-ase occur at the same site, but it seems likely.

The interpretation of the rather complicated relationship between sodium and potassium concentration and activity is not clear. If there are sites with different affinities for Na^+ and K^+ and if the rate of ATP splitting depends on how these sites are filled, then a possible explanation would be that too high a ratio of K^+ to Na^+ displaces Na^+ from the sodium sites and too low a ratio displaces K^+ from the potassium sites. However, it is not necessary to postulate the existence of sites with different affinities. If Tris were unable to reach the sites at which the alkali metal ions exert their effects, the local concentration of each of these ions would depend on the concentration of the other and mutual interaction would occur. There are other possibilities. A serious limitation of the present experiments is that because the ghosts used were highly permeable to cations it was not possible to vary the concentration on the two sides of the membrane independently. Experiments with relatively impermeable ghosts would be helpful.

It has not been possible to decide whether the 'glycoside-sensitive' and 'glycoside-insensitive' components of the ATP-ase are two separate enzymes. The ratio between the size of the two components depends on the magnesium concentration at which activity is measured (Fig. 4). Different preparations have slightly different ratios, and in a given preparation the ratio of 'sensitive' to 'insensitive' activity tends to decrease slowly if the enzyme is stored for a week at 5° C. In one experiment the fraction of activity that was sensitive to cardiac glycosides was considerably increased by hypertonicity, but this effect has not been investigated further. All attempts to extract the ATP-ase into solution have failed. Acetone, Morton's (1955) butanol procedure, various other alcohols and digitonin have been tried. Since it has not been possible to detach the glycoside-sensitive ATP-ase from the membrane, and, under the experimental conditions used, it is likely that individual transport units in the membrane remained intact and continued to pump sodium outwards and potassium inwards, it may be that any or all of the observed alterations in ATP-ase activity resulted from a primary action on some other component of the transport system. This does not affect the argument that

a close resemblance between the properties of the pump and of the ATP-ase points to a role for the ATP-ase in ion transport.

The effect of calcium in very low concentrations is remarkable, though whether it has any physiological significance is not clear. It may be significant that red cells normally contain no appreciable calcium (Ponder, 1948).

There is evidence that active movements of sodium and potassium across nerve and muscle membranes resemble those across the red cell membrane in being linked (Keynes, 1954; Hodgkin & Keynes, 1955), in being inhibited by cardiac glycosides (Matchett & Johnson, 1954; Edwards & Harris, 1957; Caldwell & Keynes, 1959), and, in nerve anyway, in depending on energy from ATP (Caldwell, 1956; Caldwell & Keynes, 1957). The question therefore arises whether an ATP-ase similar to that in red cells might exist in nerve and muscle membranes. In muscle, where it is not possible to get the membrane free from the contents of the cell, and where the contents have very considerable ATP-ase activity, this is not going to be an easy question to answer. In nerve, however, there is some evidence that such an ATP-ase may exist. Libet, in 1948, described ATP-ase activity in squid axons, and showed that the activity remained behind when the axoplasm was extruded. However, nothing is known of the effects of alkali metal ions on this enzyme, nor is it known whether the activity is in the mitochondria, or attached to the axon membrane or even in the neurilemma. Of more interest is the ATP-ase described by Skou (1957) in submicroscopic particles obtained from finely minced crab nerves. In the presence of magnesium, this ATP-ase is activated to some extent by sodium and much more by sodium plus potassium, and these properties led Skou to suggest that the enzyme may be responsible for sodium and potassium transport. Unfortunately it is not known how the particles carrying the ATP-ase activity are related to the original axon membrane.

The enzyme in the red cell membrane responsible for the splitting of ATP has been referred to throughout this paper as an ATP-ase because what is observed is the formation of inorganic phosphate. But it is quite possible that phosphate transfer occurs first, and is followed more or less immediately by hydrolysis. If phosphate transfer does occur, the phosphate must for a time be attached to some constituent of the membrane, and it might be possible to identify the phosphorylated compound if the ATP split were labelled in the terminal group with ^{32}P. However, as the intermediate might be highly unstable, or present only in trace amounts, or both, the method might not work.

SUMMARY

1. The ATP-ase activity of human red cell ghosts has been shown to consist of two components.

2. The first component requires the presence of magnesium ions but occurs in the absence of alkali metal ions and is not inhibited by cardiac glycosides. In the presence of magnesium, activity is greatly increased by small amounts of calcium but inhibited by larger amounts.

3. The second component requires the presence of magnesium and also of both sodium and potassium ions. It is completely inhibited by cardiac glycosides in concentrations sufficient to inhibit ion transport in intact red cells. Calcium ions inhibit at both low and high concentrations.

4. Experiments with certain modified cardiac glycosides suggest that the same molecular features are important for inhibition of the ATP-ase as for inhibition of the ion pump.

5. ATP-ase inhibition by very low concentrations of cardiac glycosides is prevented by raising the potassium concentration, just as, in intact cells, inhibition of the active potassium influx by low concentrations of cardiac glycosides may be prevented by raising the potassium concentration.

6. The effect of potassium concentration on the glycoside-sensitive component of the ATP-ase depends on the concentration of sodium and vice versa.

7. The close resemblance between the properties of the glycoside-sensitive ATP-ase and the ion pump suggests that they are intimately connected.

We wish to thank Dr J. B. Chappell for several helpful discussions, Professor Reichstein and Messrs Sandoz of Basle for gifts of cardiac glycosides, and the U.S. Public Health Service for a grant for apparatus.

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'APPETITE' AND THE RELATIONSHIPS BETWEEN EXPENDITURE AND INTAKE OF CALORIES IN MAN

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'Appetite' is a word which may vary considerably in its meaning with the context and the user. In this present paper the term refers simply to the natural desire for food. This desire, at least in human beings, is primarily directed towards replacing the energy which is continually being expended; the most important factor, commonly, is therefore the calorie value of the food eaten. This is not to say that other factors are unimportant. Eating by man is complicated by social customs, meal patterns, and so on. In special circumstances a craving for carbohydrate, protein or fat, or a mineral or vitamin may become evident, and it is at least theoretically possible that they may constantly exert some control over the type of diet eaten. Nevertheless, some balance between the energy value of the food eaten and the energy dissipated clearly exists. And the existence of a balance postulates the existence of some form of control.

The nature of the control which balances the intake of calories against expenditure of energy has provoked much speculation and experimentation, particularly within the past decade or so. The theories which have been proposed to explain the control of the stimuli to eat have concomitantly tended to become more refined; the range extends from the apparently old-fashioned and relatively crude idea that the mechanism involves contractions of the stomach to the more complex theories that changes in the heat balance of the body or changes in the concentration of various metabolites in the blood are the effective stimuli (e.g. Carlson, 1916; Brobeck, 1947; Kennedy, 1952–53; Mayer, 1955*b*). Whatever the mechanism, the end result is obvious. In the words of Janowitz & Grossman (1949–50) 'ingestion of food leads to inhibition of the appetitive behaviour and is accompanied by a loss of the desire to eat, . . . and (only) under pathological conditions (is) this association between the hunger state and desire for food . . . disturbed in the direction of excessive, defective or perverted tendencies'. In other words, in *physiological* conditions, where there should be no tendency to excessive or defective eating, one might expect the regulation between expenditure and intake to be a finely adjusted

mechanism and, equally, the individual to be in weight balance. The calorie value of the food eaten would, therefore, equal the calories of energy expended, with perhaps a small lapse of time in the equation of these two quantities. However, in the ordinary life of man, the regulatory mechanisms often do not appear to be particularly well adjusted. A very large number of people are frequently not in weight balance or are not regulated at their ideal weight. The commonest form of maladjustment of this regulation manifests itself in the condition of obesity which, although most prevalent in societies enjoying a high level of material prosperity, may be found in almost any region where food is not universally in short supply. Apart from the obvious maladjustment of obesity, many people who remain for long periods of time at approximately their ideal weight are probably subjectively aware that their intake of calories may vary quite markedly on separate days with little apparent relation to changes in their energy expenditure.

Only recently has there become available sufficient information to put this subjective impression to test by objective study of actual intake and expenditure of energy. Durnin (1957) briefly reported such a study. The present paper describes in detail, and discusses the implications of, an analysis which has been done on published day-to-day calorie intakes and expenditures of several groups of people living under varying circumstances.

METHODS

The daily individual intakes and expenditures of calories have been compared in six groups of people of different ages and of both sexes, who expended widely dissimilar total amounts of energy. The first group of twelve young women had a mean energy expenditure of about 2200 kcal/day (Durnin, Blake & Brockway, 1957). The second group contained twelve middle-aged housewives with a mean energy output of 2100 kcal/day (Durnin *et al.* 1957). The third group of ten male office clerks expended about 2800 kcal/day (Garry, Passmore, Warnock & Durnin, 1955). The fourth group of four male students had an expenditure of about 3200 kcal/day (Durnin & Brockway, 1959). The fifth was a group of twelve military cadets whose expenditure averaged 3400 kcal/day (Edholm, Fletcher, Widdowson & McCance, 1955), and the sixth group consisted of nineteen coal-face miners, whose average expenditure was about 3700 kcal/day (Garry *et al.* 1955). The range of these values thus encompasses the energy expenditures of the great majority of adults in this country.

Each of the sixty-nine individuals had been studied for 1 week so that there were seven consecutive daily determinations, on each subject, of both energy expenditure and calorie intake. The methods used to obtain these results were, in all cases, identical. The food intake was measured by the individual inventory method and the expenditure of energy by indirect calorimetry. Details are given in the respective publications.

The relationship between the day-to-day intake and expenditure of calories has now been analysed statistically for each individual within these groups. This has been done by obtaining the correlation coefficient (r) of the two variables and determining its significance and also by finding whether there was a significant difference between the intake (x) and expenditure (y) of calories. If the intake and expenditure of energy were perfectly adjusted

then there would be a highly significant positive correlation between the two and, since they would be equal in amount (i.e. $x-y=0$), there would be no significant difference between them. These statistical calculations have been made (1) by comparing the expenditure and intake of calories on the same day. Since it is quite possible that there may be a delay in the adjustment of intake to expenditure (Mayer, 1955*a*, *b*) and since it has been suggested that this delayed adjustment possibly occurs 1 or 2 days after the actual expenditure (e.g. Edholm *et al.* 1955), the results have been analysed (2) by comparing food intake with the energy expenditure of the previous day and again (3) with the energy expenditure of 2 days previously.

RESULTS

The correlation coefficient (r) is given in Table 1 for the ten individual subjects in the six groups, where the relationship between intake and expenditure of calories reached significance when these two variables were

TABLE 1. The correlation coefficients (r) of intake and expenditure of calories for all of the individual subjects where this reached a statistically significant level. This occurred in ten cases out of the sixty-nine subjects. Intake related to expenditure of the same day

Group	Code no. of subject	r	Significance level (%)
Young women	7	+0·818	5
Housewives	2	+0·928	1
	3	−0·850	5
	11	−0·799	5
Clerks	None	—	—
Students	2	−0·875	1
Cadets	1	−0·823	5
	6	+0·889	1
Miners	4	+0·771	5
	7	+0·839	5
	16	+0·867	5

compared for the same day. Only one of the young women, one middle-aged housewife, one cadet and three of the miners showed a significant positive correlation; in addition, two housewives, one student and one cadet had significant negative correlations of the variables. Therefore, out of the sixty-nine individual subjects, fifty-nine show no significant correlation whatever between the energy expended on any one day and the intake of calories on the same day. When the intake of calories is compared with the energy expenditure of the previous day, the number of individuals showing a significant correlation between these variables becomes reduced to 3 or 4; there is no vestige of evidence from these results that adjustment of intake to expenditure is postponed by 1 day. Intake of calories and the expenditure 2 days previously shows a similar low correlation.

The differences between the mean daily intakes ($\bar{x}$) and expenditures ($\bar{y}$)

of calories over the 7 consecutive days are presented in Table 2 in the case of those subjects where these differences are statistically significant. Some of these differences were positively significant—the intake exceeded the expenditure; negative significance signifies the reverse condition. The degree of significance is not always directly related to the amount of the difference between $\bar{x}$ and $\bar{y}$; this is explained by the fact that $\bar{x}$ might vary between 2000 and 4000 kcal and also that there might be a greater or less extent of variability in this difference—e.g. the difference of -293 kcal in student No. 4 is significant, although a difference of $+500$ kcal in one of the clerks was not statistically significant.

TABLE 2. The difference between the mean daily intake ($\bar{x}$) of calories and the expenditure ($\bar{y}$) in the individual subjects, where this was statistically significant

Group	Code no. of subject	$\bar{x}-\bar{y}$ (kcal)	Significance level (%)
Young women	4	−837	1
	9	−424	1
	12	+488	1
Housewives	8	−391	5
	10	−316	5
	12	+225	5
Clerks	3	+508	1
	4	+538	5
	5	+582	1
Students	1	−601	5
	4	−293	5
Cadets	6	+600	1
	10	+568	5
Miners	4	+992	1
	5	−610	5
	9	+883	5
	11	+870	5
	13	+962	1
	15	+991	5
	17	+484	5

DISCUSSION

A quick and even balance between intake and expenditure of energy would give a markedly positive correlation between the two and $(\bar{x}-\bar{y})$ would be non-significant. The results, summarized in Table 3, show this to be true in only four out of the sixty-nine subjects, one young woman, one middle-aged housewife and two miners. Two typical examples of this type of correlation (Type III) are shown in Fig. 1. It is obvious that the two variables do not need to follow each other identically to be significantly correlated; it is not an artificial concept. Nevertheless, it seems remarkable that such a small proportion of the total numbers of subjects (6%) should fit into this ideal pattern.

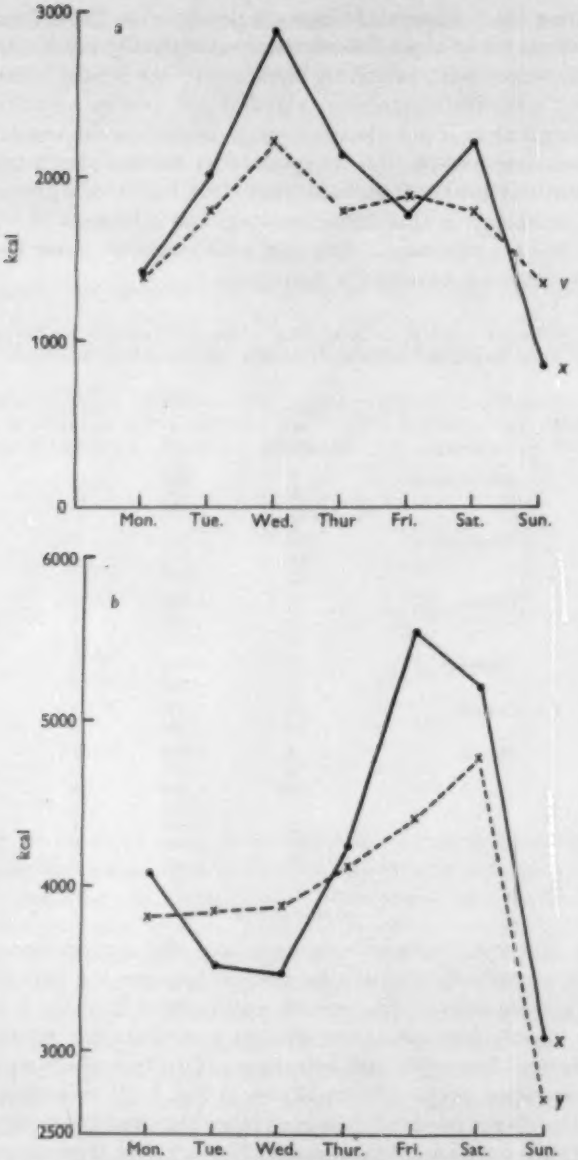

Fig. 1. Two examples showing a positive and significant correlation between daily intake (x) and expenditure (y) of calories where the mean values did not differ significantly ($\bar{x}-\bar{y}$ non-significant (n.s.)). *a*, Housewife 2: $r = +0{\cdot}928$; $\bar{x} = 1915$ kcal; $\bar{y} = 1764$ kcal. *b*, Miner 7: $r = +0{\cdot}839$; $\bar{x} = 4163$ kcal; $\bar{y} = 3940$ kcal.

On the other hand, forty-one individuals (i.e. 59 %) appear to have no significant daily correlation between their energy expenditure and food intake, although, since intake and expenditure do not differ significantly in their mean values over the week, presumably there is some form of regulation acting which prevents upset of calorie balance. Figure 2 shows two examples of this type (Type I).

TABLE 3. The differing forms of correlation* of intake and expenditure of calories shown by the individuals in each group of subjects

Correlation type	Groups: Young women	Housewives	Clerks	Students	Cadets	Miners
I	8	6	7	1	9	10
II	3	3	3	2	1	6
III	1	1	.	.	.	2
IV	.	2	.	1	1	.
V	.	.	.	.	1	1
Total	12	12	10	4	12	19

Grand total: 69 subjects

Type	r	$\bar{x}-\bar{y}$	n	% of total
I	n.s.	n.s.	41	59
II	n.s.	s.	18	26
III	+s.	n.s.	4	6
IV	−s.	n.s.	4	6
V	+s.	s.	2	3

* s. = significant; n.s. = non-significant; +s. = positively significant; −s. = negatively significant.

Figure 3 gives two examples of the eighteen individuals (26 %) classified as Type II, where there is no significant correlation between daily calorie expenditure and intake but where the mean values of the two series of measurements differ significantly. This difference shows an insufficiency of calorie intake in seven individuals and an excess in the other eleven, so that presumably if this were a process continuing over a sufficiently long period, these individuals would lose or gain weight, respectively. An analysis of the separate subjects in this category is not very rewarding. Three of the four women with a calorie intake less than output were plump in physique and might possibly have been deliberately restricting their food intake. The other woman and all the men, with one exception (miner 17), were normal in build, neither thin nor fat, and were eating their normal diet. If the individuals were obese, then this might satisfactorily explain either a positive or a negative difference between $\bar{x}$ and $\bar{y}$—they might still be gaining weight or they might be trying to lose weight. Where there is no evidence of other than a healthy distribution of body tissue, theorizing about the significant difference between $\bar{x}$ and $\bar{y}$ is difficult.

Four individuals (Fig. 4) have a significant *negative* correlation between

intake of energy and output; again, however, as with the four subjects in Type III, with no difference between the mean values for the two measures, (Type IV). It is theoretically possible that an individual might have fluctuations in daily energy expenditure of such an order that, on the days

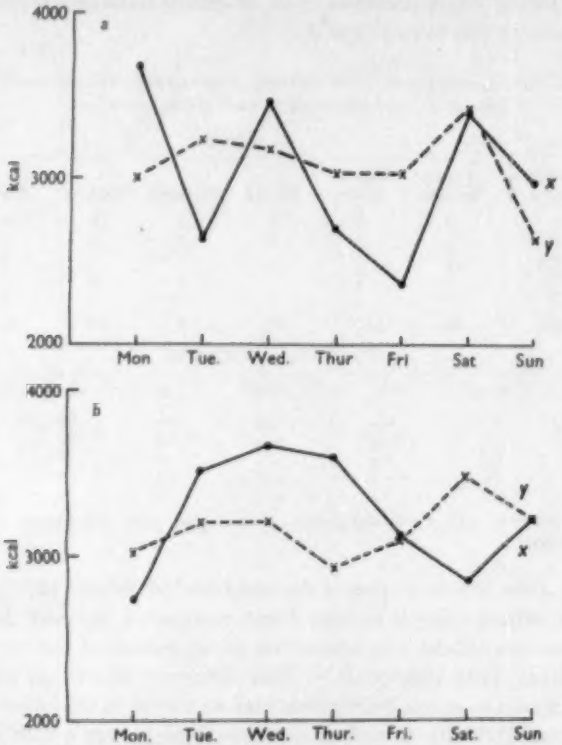

Fig. 2. Two examples where individuals showed no significant correlation between daily intake (x) and expenditure (y) of calories and where ($\bar{x}-\bar{y}$) was non-significant. *a* Cadet 8: $r = +0{\cdot}196$; $\bar{x} = 3034$ kcal; $\bar{y} = 3080$ kcal. *b* Clerk 8: $r = -0{\cdot}274$; $\bar{x} = 3248$ kcal; $\bar{y} = 3168$ kcal.

when this was at a high level, there might be insufficient time or inclination to eat enough, and the deficit might be made up on a subsequent day. This would result in a form of graph such as that seen in Fig. 4, but the explanation is not valid in any of the four cases here. If there is a satisfactory interpretation it is difficult to imagine one agreeing with present physiological explanations of appetite control.

Only two individuals fall into Type V, where there is a high positive correlation between intake and expenditure but a significant excess of

intake over output (Fig. 5). This would suggest a control of appetite regulated consistently but at the wrong level. A mechanism such as this would explain those cases of obesity which develop over a period and then eventually become stable.

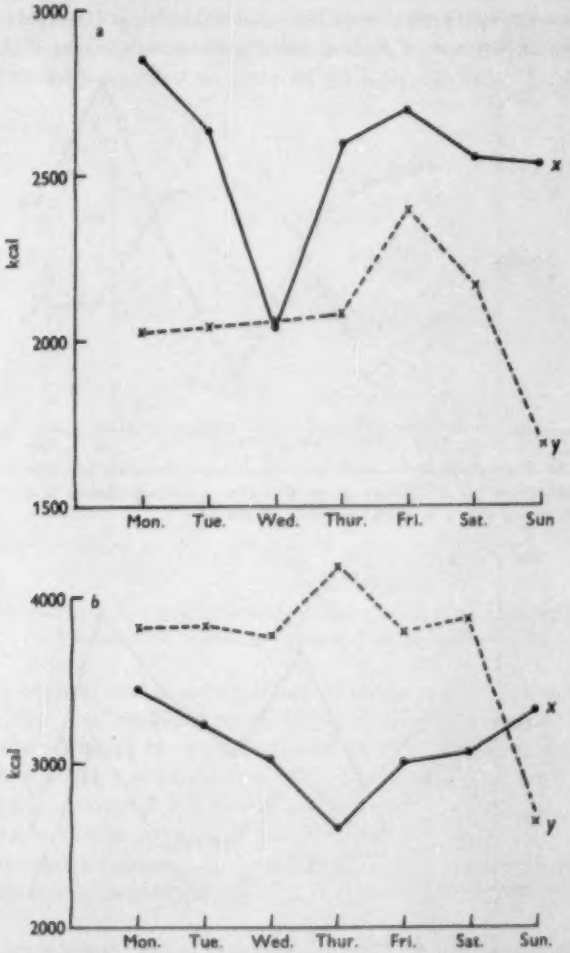

Fig. 3. Two examples showing no significant correlation between daily intake (x) and expenditure (y) of calories but where ($\bar{x}-\bar{y}$) was significant. In (a), there was an excess of intake over expenditure and in (b) a deficiency. *a* Young woman: $r = +0{\cdot}156$; $\bar{x} = 2555$ kcal; $\bar{y} = 2067$ kcal; $\bar{x}-\bar{y}$ significant, $P < 0{\cdot}01$. *b* Miner 5: $r = -0{\cdot}535$; $\bar{x} = 3089$ kcal; $\bar{y} = 3699$ kcal; $\bar{x}-\bar{y}$ significant, $P < 0{\cdot}05$.

The findings analysed above, if they represent in a reasonably accurate way the normal relationship between expenditure and replacement of calories in human beings, make total acceptance of any of the current explanations of appetite control unsatisfactory. That the results are not totally unrepresentative is demonstrated by some other relevant publications; for example, the results of Booyens & McCance (1957) show similar trends, even in the case of such a well-regulated subject as R. A. McC.

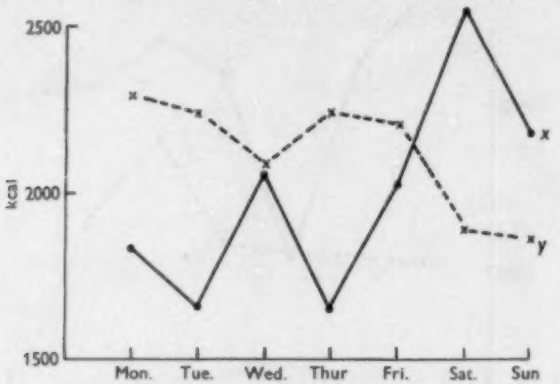

Fig. 4. An illustration of a *negative* and significant correlation between intake (x) and expenditure (y) of calories where ($\bar{x}-\bar{y}$) was non-significant (n.s.). Housewife 3: $r = -0{\cdot}850$; $\bar{x} = 1999$ kcal; $\bar{y} = 2120$ kcal.

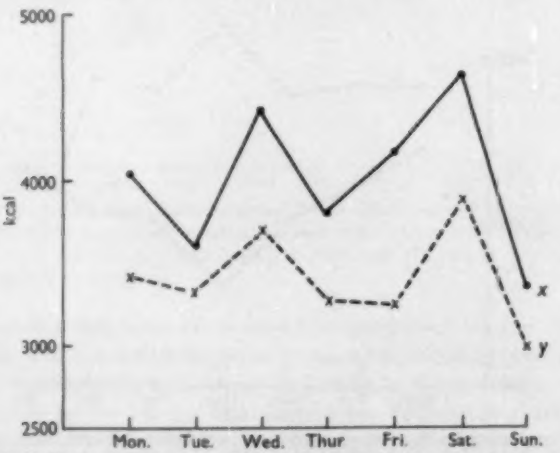

Fig. 5. An individual subject where intake (x) of calories was significantly and positively correlated with expenditure (y) but where the mean values differed significantly. Cadet 6: $r = +0{\cdot}889$; $\bar{x} = 3992$ kcal; $\bar{y} = 3396$ kcal; ($\bar{x}-\bar{y}$) significant, $P < 0{\cdot}01$.

The values analysed in this paper bear little relation to the much more uniform distribution found in studies on lower mammals (e.g. Gasnier & Mayer, 1939). Figure 6 shows the scatter of the results (for the young women and the housewives) where intake on the abscissa is plotted against expenditure as the ordinate. The other groups show a similar scatter. And the relationship cannot be elucidated by postulating a delay in adjustment, as did Edholm *et al.* (1955). In fact, their results, when analysed in the above manner, show no grounds for inferring delay in operation of control.

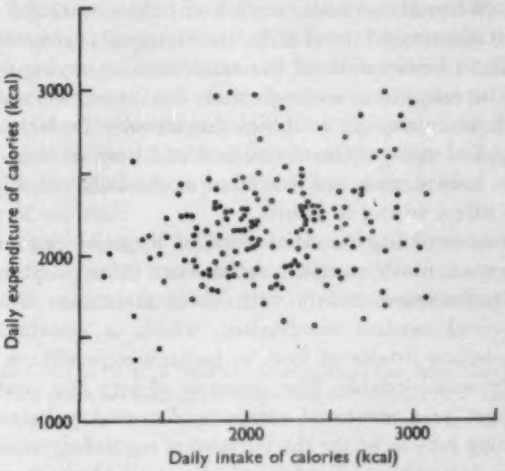

Fig. 6. Scatter of daily intake of calories plotted against expenditure (same day) for 7 consecutive days in 24 young and middle-aged women.

Both the subjective and the objective evidence analysed here lead to the conclusion that the mechanism whereby our appetite is satisfied is by no means nicely adjusted to expenditure of energy. The high incidence of obesity when there are no restraints on the availability of food would alone suggest a control relatively insensitive to precise regulation of calorie balance, as also would the low correlation of intake and expenditure shown in the above analysis. In contrast, there is clearly some functioning control since a long-term approximate calorie balance is generally attained in man.

It may be inferred, then, that perhaps a double mechanism controls appetite, (1) a gross, short-term regulation and (2) a long-term one, operating with a delay of days, weeks or occasionally months. The usually dominant control, the short-term control, may be a simple physical mechanism, such as the state of distension or motility of the stomach or

possibly also of the duodenum and proximal jejunum. A control such as this would satisfactorily interpret the large variability shown in the intake of calories by an individual, since the same bulk of food may have a very different calorie content. The temporary efficacy of high-bulk, low-calorie diets in the treatment of obesity would also be explained. Studies on babies described by Yorston & Hytten (1957) give added support for such a theory. They found that a certain number of babies fed on breast milk cried soon after a feed although there were no indications of underfeeding; the signs were similar to those of other similarly fed babies suffering from underfeeding. When these first mentioned babies were fed with similar quantities of reconstituted dried milk, the 'hunger' crying stopped. When returned again to breast milk of the same amount crying recommenced. Radiological investigation showed that the stomach and duodenum emptied much more rapidly with the breast-milk feed than with dried milk. The physical state of the stomach would here seem to be the determining factor; babies, also, are free from most of the other psychological factors which affect eating in adults.

The factors determining the satisfaction of 'appetite' would thus usually seem to be, in man, much cruder in action than those proposed by workers who have experimented mainly with lower mammals. The existence of a finely triggered control mechanism, which is clearly ineffectual in adapting immediate intake of food to balance expenditure of energy, is physiologically unacceptable. The presence of any fine control therefore becomes suspect as a means of attaining day-to-day balance. Yet it is possible that one may exist for the purpose of regulating intake after short periods (e.g. a day or part of a day) of strenuous physical exercise, where large debts of calories may require to be repaid. However, almost certainly, there must be another, long-term control exerted over appetite and some metabolic, possibly lipostatic, factor must act as a further, superadded influence.

SUMMARY

1. Relationships between the intake and expenditure of calories in man have been determined. Measurements, previously published, of the daily intake and expenditure of energy during a period of 7 consecutive days by each of sixty-nine individual subjects have been analysed statistically. The subjects were adults of both sexes, with a range in age from young to elderly and in mean daily energy expenditure from 2000 to 4000 kcal.

2. In each individual the correlation coefficient (r) was determined for (1) intake of calories and the expenditure of the same day. Since there may be a delay in the adjustment of intake to expenditure, r has also been calculated where the intake has been related to the energy expenditure (2)

of the previous day and again (3) with the energy expenditure of 2 days previously. The significance or otherwise of the difference between the mean daily calorie intake ($\bar{x}$) and expenditure ($\bar{y}$) (over 7 days) has also been analysed.

3. A quick and even balance between intake and expenditure would give a markedly positive correlation between the two (r positive and significant) and $\bar{x}$ minus $\bar{y}$ would be non-significant. This was the case in four out of the sixty-nine subjects. In forty-one subjects (59 %) r was not significant and ($\bar{x}-\bar{y}$) was also not significant; that is, intake and expenditure were balanced over the 7 days, although there was no significant correlation between the two from day to day. Eighteen individuals (26 %), showed r to be non-significant but there was a significant difference between mean intake and expenditure of calories (seven subjects had an insufficient intake of calories and eleven an excess). A significant *negative* correlation for r was present in four individuals, where $\bar{x}-\bar{y}$ was not significant. Two subjects had a significant and positive r, but a significant excess of intake over output of calories.

4. These results make it unlikely that most of the current theories on the control of 'appetite' can be directly applied to man; the factors regulating this control from day to day would seem to be much more gross in action.

I am much indebted to Dr R. A. Robb, Department of Statistics, University of Glasgow, for advice and help with the statistical analyses and to Professor R. C. Garry for his helpful comments. The work formed part of a research project financed by the Scottish Hospital Endowments Research Trust.

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RELATIVE PENETRABILITY OF HAIR FOLLICLES AND EPIDERMIS

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The skin protects the body against foreign chemicals by restricting their passage through the epidermis; diffusion of common organic molecules through rabbit epidermis is two to three orders of magnitude slower than through dermis (Treherne, 1956). Such diffusion as does occur might be through the epidermis itself or through the defect structures, the hair shafts and sweat glands. Argument about this has hinged on the histological localization of applied chemicals (Rothman, 1954). The present study is an attempt to resolve the problem by measuring the passage through each route separately.

METHODS

A 20–30 kg pig was anaesthetized with dial-urethane. The flank was washed and its hair clipped very short. Seven 1 in. (2·5 cm) circles were drawn on the skin, corresponding to the holes in a metal covering plate (Fig. 1). Tri-*n*-butyl phosphate (TBP) containing ^{32}P of radioactivity 0·2 mc/mole and 2 % of a fluorescent dye (Uvitex W, Ciba) was placed on the skin within these circles with a fine entomological pin. Approximately 0·1 μmole of TBP was placed in each circle in 20–30 separate patches. In four of the circles the TBP was placed, as far as possible, so as to avoid the hair follicles and in the other three the hair follicles were deliberately filled with TBP. The number of hair follicles filled by the fluorescent dye was counted under ultra-violet light. A mica cover was sealed over each skin circle by surrounding adhesive tape and the metal plate already mentioned was pressed down over the entire set of circles to prevent movement during respiration. Each circle in turn was exposed to an end-window Geiger counter at 20 cm distance. These measurements continued for 4–6 hr, care being taken that the geometry of the system was not disturbed. At the end of this time the number of hair follicles covered by fluorescent dye was again measured. A dental X-ray film was pressed into contact with each mica cover and an autoradiograph of the ^{32}P on the skin obtained. The mica covers were removed and the fluorescent dye on each skin circle was photographed. Finally, the animal was killed and each skin circle was dissected out, digested in nitric acid and its ^{32}P content determined. In a few experiments the pig was killed 1 hr before the β-emission measurements were concluded.

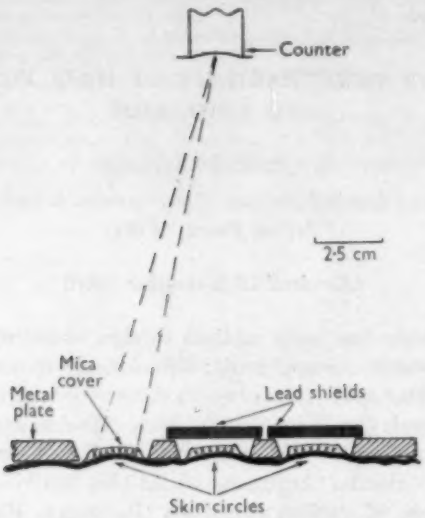

Fig. 1. Diagram of the apparatus used; the mica cover is indicated. Note the small angle subtended by the TBP at the Geiger counter. × × × × = TBP.

RESULTS

Each tiny (approx. 5 nmole) drop of TBP placed on the skin spread to cover approx. 0·03 cm^2, whether the drop involved a hair shaft or not. When a hair shaft was covered by TBP a bright spot of fluorescence could be seen at the entrance of the hair into the epidermis, indicating the presence of a pool of TBP. Many of these pools were still present at the end of the experiment. The hairs themselves fluoresced brightly, and by the end of the experiment fluorescence of the intra-epidermal part of the hair could be seen through the nearly transparent epidermis, showing that the marker dye had penetrated into the hair shaft.

In four out of the seven skin circles used in each experiment an attempt was made to avoid the hair shafts during application of the TBP. This was not completely successful; the marker dye showed that the TBP reached some hair shafts immediately, and spread to a few more by the end of the experiment. The number was, however, small compared to those filled deliberately in the other three skin circles (Table 1).

The β emission from the ^{32}P in each skin circle fell during the experiment; the rate of fall was constant after the first hour (Fig. 2). The fall of β emission reflects a movement of ^{32}P out of the counting field of the Geiger tube. As the alkyl phosphates hydrolyse very slowly at 40° C (Cavalier, 1898) and do not oxidize at all (Kosolapoff, 1950) it is very

likely that the ^{32}P moved in association with TBP; thus the fall in β emission indicates a movement of TBP. The fall ceased immediately the animal was killed (Fig. 2). Since evaporation of TBP through hypothetical leaks in the mica-adhesive tape seal or diffusion of TBP deep into the dermis would not be immediately affected by the animal's death, these movements were assumed to be negligible. There was no apparent lateral movement of the TBP on the skin surface to be seen in the photographs

TABLE 1. Average number of hair follicles covered by TBP in each skin circle when follicles were avoided (*A*) or deliberately filled (*B*)

Time after application (min)	*A*	*B*
10–30	3·3 (0·4)	25·0 (0·8)
240–300	0·3 (0·5)	23·7 (0·8)

Values are derived from 40 (*A*) or 30 (*B*) skin circles: S.E. of means in parentheses.

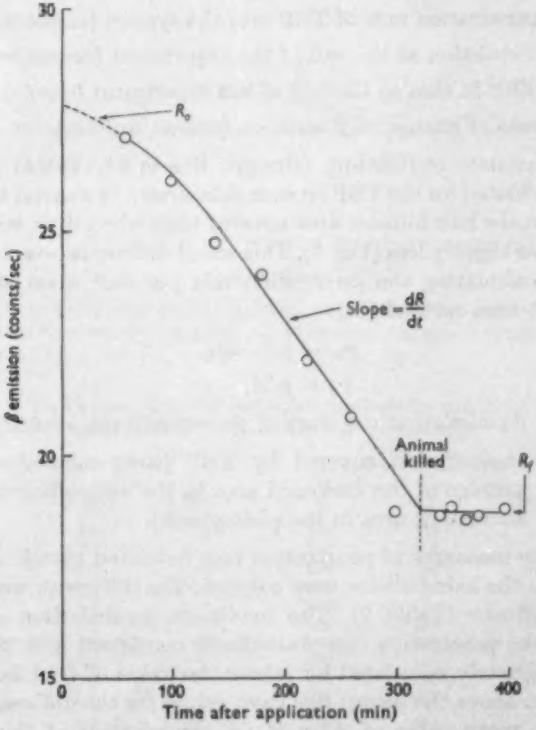

Fig. 2. β emission from a skin circle to which TBP has been applied. Symbols are those defined in the text.

or autoradiographs taken at the end of the experiment, so that screening by the metal covering plate may also be discounted. Changes in thickness of the TBP on the skin surface, or movement within the epidermis, are unlikely to have much affected the emission, for the distances involved are of the order of 10^{-2} mm and the half-thickness for ^{32}P is approx. 1 mm (Faires & Parkes, 1958). It therefore follows that the reduction in β emission was due to the only remaining route, the passage of TBP through the epidermis or hair shafts and into the systemic circulation, and it is on this assumption that the following results are construed.

^{32}P distributed throughout the body would be virtually completely screened from the Geiger tube so that the rate of fall of β emission should be proportional to the rate of penetration of TBP into the systemic circulation:

$$p = \frac{m}{R_f} \times \frac{dR}{dt},$$

where p = penetration rate of TBP into the system (nmole/min),

R_f = β emission at the end of the experiment (counts/sec),

m = TBP in skin at the end of the experiment (nmole),

dR/dt = rate of change of β emission (counts/sec/min).

During dynamic equilibrium (straight line in Fig. 2) this penetration rate was calculated for the TBP on each skin circle. It was not significantly greater when the hair follicles were covered than when they were avoided; in fact it was slightly less (Fig. 3). This small difference was investigated further by calculating the penetration rate per unit mass of TBP (p_m) and per unit area covered (p_a):

$$p_m = pR_f/mR_o,$$

and

$$p_a = p/A,$$

where R_o = β emission at the start of the experiment (counts/sec),

A = area of skin covered by TBP (cm^2; calculated from the average of the darkened area in the autoradiograph and the fluorescent area in the photograph).

Both these measures of penetration rate indicated that it was slightly slower when the hair follicles were covered. The difference was, however, barely significant (Table 2). The maximum contribution of the hair follicles to the penetration rate statistically consistent with these results was approximately calculated by taking the value of B–A in Table 2 at 2·5 × the S.E. above the mean; this gave values for the difference of 2 and 13% of the mean value of A or B, i.e. the addition of the extra hair follicles in group B did not increase the penetration rate by more than

15%, or approx. 20 pmole/min (cf. Fig. 3). The maximum follicular transport possible was therefore 1 pmole/min/follicle; it is more probable that involvement of the hair follicles actually reduced the penetration of the TBP.

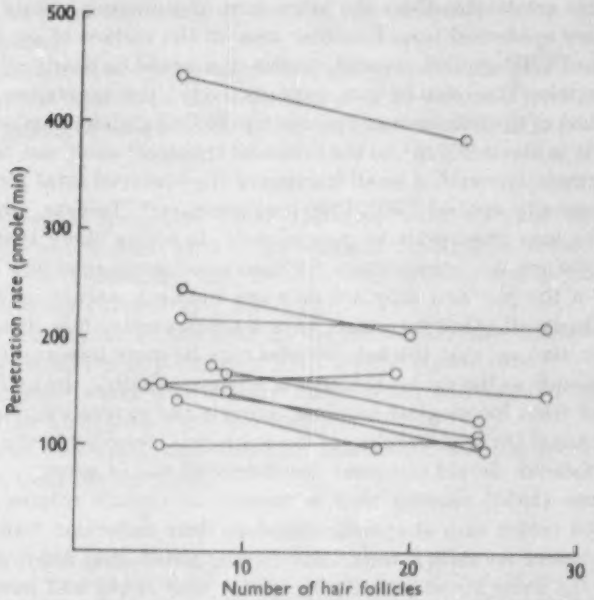

Fig. 3. The effect of covering hair follicles with TBP on its penetration rate. Each point is the mean from 3–4 skin areas; the lines join points obtained on the same animal.

Table 2. Penetration of TBP through skin in which the hair follicles were either avoided (*A*) or deliberately covered (*B*)

p_m (kmin^{-1})			p_s (pmole/min/cm^2)		
A	*B*	*B*–*A*	*A*	*B*	*B*–*A*
2·4	2·1	−0·3	190	120	−70
1·5	1·3	−0·2	180	220	+40
1·1	1·1	0·0	170	180	+10
0·7	1·1	+0·4	90	140	+50
1·6	1·3	−0·3	200	170	−30
1·6	1·2	−0·4	140	120	−20
2·0	1·6	−0·4	290	240	−50
2·5	1·8	−0·7	530	440	−90
1·4	1·4	0·0	260	200	+20
1·1	1·0	−0·1	120	150	+30
Mean 1·6	1·4	−0·20 (s.e. 0·09)	218	204	−13 (s.e. 16)

Each value is the mean of four (*A*) or three (*B*) skin areas in a single experiment.

DISCUSSION

There was no indication in these experiments that any TBP passed through the hair follicles to the circulation. The hair follicles appeared to be no more penetrable than the same area of epidermis. Since there is vastly more epidermal than follicular area on the surface of pig skin, the transport of TBP applied generally to the skin would be nearly all through the epidermis. This can be put quantitatively: the maximum possible contribution of the follicles was 1 pmole/min/follicle and the number of hairs on pig skin is about 20/cm², so the follicular transport could not be greater than 20 pmole/min/cm², a small fraction of the observed total penetration rate of generally-applied TBP, 1300 pmole/min/cm² (Tregear, 1960).

How far may this result be generalized? It seems likely that in man hair follicles are also unimportant, for their histological structure is similar to those of the pig, and they are no more frequent, except on the hairy areas. Nearly all other mammals have a much greater hair density than the pig or man, so that the hair follicles may be more important; indeed, in species such as the rabbit the area of epidermis within the hair follicles, calculated from histological sections, exceeds the exposed surface of the skin, so that if the penetrability of the epidermis is similar in the two sites the hair follicles should represent the dominant site of entry.

Treherne (1956) showed that a variety of organic solutes in water penetrated rabbit skin at speeds related to their molecular diameter and their oil:water solubility ratio, and he suggested that they all passed through the same structure. If this is also true of pig and human skin, the results obtained with TBP, a stable aliphatic ester of no known special properties, should apply to other chemicals.

The evidence on which it has been suggested that foreign chemicals pass through the skin predominantly via the hair follicles is histological. It has been observed that many materials placed on the skin surface reach the lumen of the follicles (MacKee, Herrmann, Baer & Sulzberger, 1945; Axelrod & Hamilton, 1947; Sulzberger, 1948; Borelli & Metzger, 1957), although this is not always the case (Blank, Griesemer & Gould, 1958). This is a necessary but not a sufficient condition for penetration via the hair follicles to occur. Concentrations of chemical in the dermis around the hair follicles have also once been observed (MacKee *et al.* 1945); this showed that the chemicals used (water solutes of various types) had probably passed through the lining epithelium or sebaceous glands. It did not, however, show how much had passed through. In general, histology, unless combined with a rate study, cannot demonstrate the relative importance of competitive routes of penetration.

In man, though not in the pig, a further defect in the epidermis is

provided by the sweat glands. Since these glands are several times more numerous on the palms than on the body surface in general (Kuno, 1956), and the palms are stated to be less penetrable than other parts of the skin (Herrmann, 1945; Shelley & Melton, 1947), it is unlikely that the glands represent an important route of entry of foreign chemicals on sites other than the palms.

In conclusion, it seems probable that the protective impenetrability of human skin is limited by the structure of the epidermis itself, and not by sweat glands or hair follicles acting as holes punched in it.

SUMMARY

1. Tri-*n*-butyl phosphate (TBP) labelled with ^{32}P was applied in small drops to the flank skin of an anaesthetized pig in seven circular areas. In four of these the hair follicles were avoided and in three they were deliberately covered with TBP.

2. The penetration of the TBP to the circulation was estimated from the steady reduction in β emission from the ^{32}P on the skin.

3. This penetration was slightly faster when the hair follicles were avoided than when they were covered.

4. It was concluded that the hair follicles of the pig are no more penetrable than the epidermis.

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THE MEASUREMENT OF SODIUM AND POTASSIUM ACTIVITIES IN THE SQUID AXON BY MEANS OF CATION-SELECTIVE GLASS MICRO-ELECTRODES

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(*Received* 23 *November* 1960)

Most techniques for measuring intracellular sodium and potassium give varied results largely because the tissue has to be damaged before chemical analysis. Some methods have an additional disadvantage of allowing too much sodium and potassium exchange or contamination from the extracellular space. Also, for practical purposes, investigators are forced to assume that all the sodium and potassium is uniformly distributed and physico-chemically 'free' in the cellular water. Clearly, a technique that provided a method for quick direct measurement of 'free' intracellular sodium and potassium without damaging the cell would have great theoretical importance.

In part the cation-selective glass micro-electrodes (Hinke, 1959) fulfil the requirements for this ideal method. They provide a technique for following intracellular sodium and potassium without causing too much damage; they measure only 'free' cations (activities), so that an estimate of the bound sodium and potassium in the protoplasm can be made.

The aim of the experiments in this paper was to measure sodium and potassium activities inside and outside the isolated giant axon of the squid in order to relate the cation equilibrium potentials to the resting and action potential of the cell membrane. The net fluxes of sodium and potassium across the membrane during stimulation were also measured.

METHODS

Cation-selective glass micro-electrodes. The design of the Na and K glass electrodes (Fig. 1) was similar to those constructed earlier (Hinke, 1959), except that the exposed tip was 70–90 μ in diameter and 2–4 mm long, and the shaft was 6 cm long with the lower 3 cm not exceeding 150 μ in diameter.

The glass was supplied by Eisenman, Rudin & Casby (1957) and was worked into tubing 2–4 mm outside diameter. Oxyacetylene was required to melt the Na glass, but air and gas

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was satisfactory for the K glass. The composition of the glasses according to Eisenman is listed in Table 1. This composition is not strictly correct for the finished micro-electrodes, since soda is lost each time the glasses are melted. Microcapillaries were pulled by hand from the 2–4 mm stock tubing.

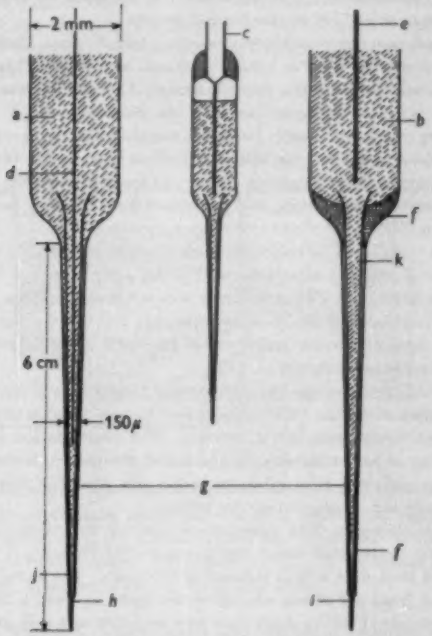

Fig. 1. Diagram of Na and K micro-electrodes. *a*, 0·1 N-NaCl, pH 8·0; *b*, 0·1 N-KCl, pH 8·0; *c*, gold-plated jack plug; *d*, 50 μ Ag-AgCl wire; *e*, 300 μ Ag-AgCl wire; *f*, dental wax; *g*, high-resistant glass; *h*, Na-sensitive, and *i*, K-sensitive glass, exposed tips 2–4 mm × 70–90 μ diam.; *j*, glass-to-glass seal; *k*, air space; numbers show outside measurements.

TABLE 1. Composition of cation-selective glass

	SiO_2 (mole %)	Al_2O_3 (mole %)	Na_2O (mole %)	CaO (mole %)
Na glass	71	18	11	—
K glass	63	7	27	3

The main body of the electrode was pulled from high-resistant L-I glass (General Electric Co.) by means of a micro-electrode puller. A microcapillary was matched according to its diameter with an appropriate L-I glass micropipette and fed down the latter until sufficient length extended beyond the tip of the L-I pipette. The rest of the construction was done with a modified de Fonbrune microforge. This consisted of two micromanipulators, one for holding the micropipette and the other for a V-shaped microforge wire (platinum-iridium alloy). The micropipette was mounted vertically, illuminated from behind, and observed at a right angle through a binocular microscope.

The next step for the Na electrode was to heat the last few millimetres of the L-I pipette until it fused to the wall of the inside Na glass capillary. After this area had cooled slowly, the pipette was filled to the top with 0·1 M-NaCl (pH 8·0). Then pressure was applied at the top of the pipette until a bead of liquid formed at the open tip (this procedure reduced air entry when the tip was heated). The electrode was returned to the microforge and the exposed tip of Na glass sealed off at the desired length.

A glass-to-glass seal was not possible between L-I and K glass. Instead, chips of dental wax were dropped down the pipette until the shank was filled. This area was heated to make the upper wax seal between the two capillaries. The pipette was filled with 0·1 M-KCl (pH 8·0), and after applying pressure, as with the Na electrode, it was returned to the microforge for sealing of the tip. Lastly, wax was melted on to the tip of the microforge wire, which was moved close to the L-I tip allowing molten wax to run between the two capillaries. Figure 1 illustrates how a column of air was trapped between the upper and lower wax seals. The air served as insulation and prevented fluid entering from below if the lower wax seal broke down.

The electrode was completed by sealing in with Araldite a jack plug which had a Ag-AgCl wire soldered to it. In the Na electrode, a 50 μ Ag-AgCl wire was fed down the micro-capillary close to the active tip. This refinement was not possible in the K electrode since the upper wax seal blocked vision of the capillary opening.

The resistance of these micro-electrodes was of the order of $10^9\ \Omega$ compared to 10^{10}–$10^{11}\ \Omega$ for the smaller electrodes constructed in 1959.

Excluding accidents, the average life was about 6 months for a Na micro-electrode, and 1 month for a K micro-electrode. When they were tested daily in standard solutions, the electrodes gave remarkably constant potentials. If a prolongation in response time and relatively large change in cation selectivity was noted, the electrode was discarded. Usually an electrical leakage occurred soon after, reducing the potential response from 58 mV to 25 or 30 mV for a unit log. change in concentration.

Conventional micro-electrodes. The micro-electrodes for recording membrane and action potential were similar to the final design recommended by Hodgkin & Katz (1949*a*), except that they were filled with 0·58 M-KCl instead of sea water. The main features were a 30 μ silver wire extending from the shank almost to the open tip, and a 300 μ Ag-AgCl wire in the body of the electrode. This Ag-AgCl wire was soldered to a jack plug which was sealed in with Araldite. The purpose of these permanent jack plugs was to permit quick exchange of the electrodes on the input leads as well as to reduce contact potentials.

Electrical system. Two screened input leads were fitted with sockets to match the electrode plugs. One input was for recording action potentials, and the circuit included a cathode-follower valve, direct-coupled pre-amplifier (Copeland, 1952), and oscilloscope. The other input went directly to a 'Vibron' electrometer model 33B (Electronic Instruments Ltd) and was used to measure membrane potential, Na and K electrode potentials. A calibrator was placed between earth and the reference electrode in the recording cell. It served to calibrate the oscilloscope tube scale, or as a backing off unit for the Vibron. The cathode follower, micromanipulators, recording cell and microscope were housed in a large aluminium screening box.

Recording cell. The Perspex recording cell was essentially similar to one used by Hodgkin & Huxley (1945) except for a built-in Ag-AgCl reference electrode in saturated KCl which made contact with the bath through a fine porcelain plug (Fig. 2). A platinum stimulating electrode was placed in each chamber. The sliding glass partitions were well greased with petroleum jelly so that contact between chambers was localized at the groove where the axon penetrated. With this system, the threshold strength of stimulus was usually less than 60 mV and rarely over 100 mV as measured on the oscilloscope.

Solutions. Sea water was used for all dissections, but either sea water or artificial sea water (Hodgkin & Katz, 1949*a*; Hodgkin & Keynes, 1955) was used in the recording cell. The average compositions are listed in Table 2.

Experimental procedure. Giant axons were obtained from the hindmost stellar nerve of *Loligo forbesi*. The methods of dissection, cleaning, mounting in the recording cell, and impaling with an electrode were similar to those used by Hodgkin & Huxley (1945). The squid was killed and eviscerated on board ship and its mantle placed in ice-cold sea water (Caldwell, Hodgkin, Keynes & Shaw, 1960). Experiments were usually started after the axon was dissected and cleaned, but occasionally axons were stored during the night in sea water at 0° C. If these axons conducted an impulse the following morning, an experiment was carried out.

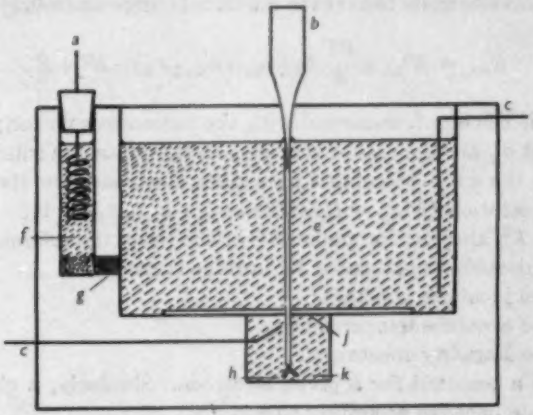

Fig. 2. Diagram of Perspex recording cell with built-in Ag-AgCl reference electrode, (*a*). A giant axon is shown cannulated and mounted. *b*, cannula; *c*, stimulating Pt wires; *e*, main chamber; *f*, saturated KCl; *g*, porcelain plug; *h*, Pt wire weight; *j*, sliding glass partition; *k*, lower chamber.

TABLE 2. Approximate composition of external fluids

Substance	Sea water* (mole/kg H_2O)	Artificial sea water (mole/l.)
K^+	0·0105	0·0105
Na^+	0·470	0·465
Ca^{2+}	0·010	0·011
Mg^{2+}	0·055	0·055
Cl^-	0·550	0·605
SO_4^{2-}	0·030	—
HCO_3^-	—	0·0025
pH	7·5–8·0	8·0

* Average salinity 3·50 % for November, December, January, 1959–60. Composition calculated according to Webb (1939).

All electrode measurements were referred to the built-in reference electrode. The cation glass electrodes were tested in standard solutions before and after each experiment. The potential in the bath solution was recorded for each electrode before inserting it into the axon.

At a given depth in the axon, membrane resting and action potentials were recorded before and after Na and K glass electrode potentials. A complete set of measurements, therefore, involved four insertions: (*a*) 0·58 M-KCl electrode; (*b*) Na glass electrode; (*c*) K glass electrode; (*d*) 0·58 M-KCl electrode. This procedure usually required five minutes.

RESULTS

Calibration of Na and K glass micro-electrodes

The procedure for finding the Na and K concentration of an unknown solution with cation-selective electrodes was suggested by Eisenman, Rudin & Casby (1958). If the $[H^+]$ is sufficiently low not to interfere, a given Na glass electrode behaves in Na–K mixtures according to eqn. (1):

$$E_{Na} = E^0_{Na} + \frac{RT}{F} \ln (a_{Na} + k_{NaK} a_K) - E^0_j + E_j, \quad (1)$$

where E_{Na} is the e.m.f. measured with the unknown solution;

a_{Na} and a_K are the cation activities in the unknown solution;

E^0_{Na} is the e.m.f. calculated for a given electrode from its response to a standard solution ($E^0_{Na} = E_{Na}$ when $a_{Na} + k_{NaK} a_K = 1$);

E_j and E^0_j are junction potentials at the tip of the reference electrode (usually neglected if a saturated KCl bridge is used);

R is the ideal gas constant;

T is the absolute temperature;

F is the Faraday constant;

k_{NaK} is a constant for a given electrode. Similarly, a given K glass electrode behaves according to eqn. (2):

$$E_K = E^0_K + \frac{RT}{F} \ln (a_K + k_{KNa} a_{Na}) - E^0_j + E_j. \quad (2)$$

If E^0_j and E_j are neglected, the constants E^0_{Na}, E^0_K, k_{NaK} and k_{KNa}, in eqns. (1) and (2) can be calculated from the measured potentials E_{Na} and E_K in standard solutions. The four standards were 0·1 M and 0·01 M-NaCl and KCl buffered to pH 8·0 with tris-(hydroxymethyl) aminomethane. The mean activity coefficients for the standards were taken from Robinson & Stokes (1959).

In practice, the electrodes did not always give 58 mV change for a unit log. change in activity. At 18°C the calibration curves gave slopes between 52 and 57 mV for the K electrodes, and between 57 and 62 mV for the Na electrodes. Because of these deviations from the theoretical constant, $2{\cdot}3\,RT/F$, this was replaced by an empirical slope constant S actually obtained from the standard solutions. Therefore, eqns. (1) and (2) can be rewritten as eqns. (3) and (4) (ignoring junction potentials):

$$\text{Na glass electrode, } E_{Na} = E^0_{Na} + S_{Na} \log_{10} (a_{Na} + k_{NaK} a_K); \quad (3)$$

$$\text{K glass electrode, } E_K = E^0_K + S_K \log_{10} (a_K + k_{KNa} a_{Na}). \quad (4)$$

One Na electrode was used for all but the first four experiments. Since

the daily calculated value of k_{NaK} was consistently 0·005 or less, eqn. (3) was simplified to

$$E_{Na} = E^0_{Na} + S_{Na} \log_{10}(a_{Na}). \quad (5)$$

Both Na and K electrodes responded to some extent to [H+]. Figure 3 demonstrates the degree of interference of pH for a Na glass electrode tested in NaCl solutions (0·20–0·01 M). For clarity, only pH 8·0 and 5·9 curves are shown. The pH 7·0 and 7·5 curves deviated only slightly from

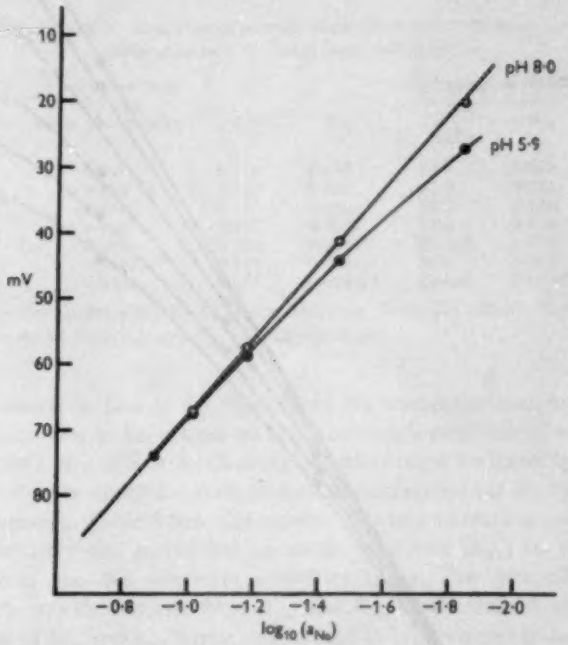

Fig. 3. Response of the Na micro-electrode to NaCl solutions at pH 8·0 and pH 5·9.

the pH 8·0 curve and only in the region where a_{Na} was low. The degree of interference of pH for the K electrodes in pure KCl solutions of the same concentration range was similar. No corrections were made for H^+ ion, since the pH of sea water and artificial sea water was 8·0, and the pH of squid axoplasm was greater than 7·0 (Caldwell, 1958). If an appreciable error is introduced by ignoring pH effects in the axoplasm, Fig. 3 suggests that the error will be such as to make the calculated a_{Na} (or a_K) too large.

The k_{KNa} values for the K electrodes were usually between 0·1 and 0·4. This meant that the K electrodes were only satisfactory for solutions where [K^+] was high or [Na^+] was low. Such a situation existed in the

axoplasm but certainly not in the bath solution. Therefore, no attempt was made to determine the $(a_K)_o$ of the bath solution.

Since eqn. (4) was determined from only three standards, 0·01 M,

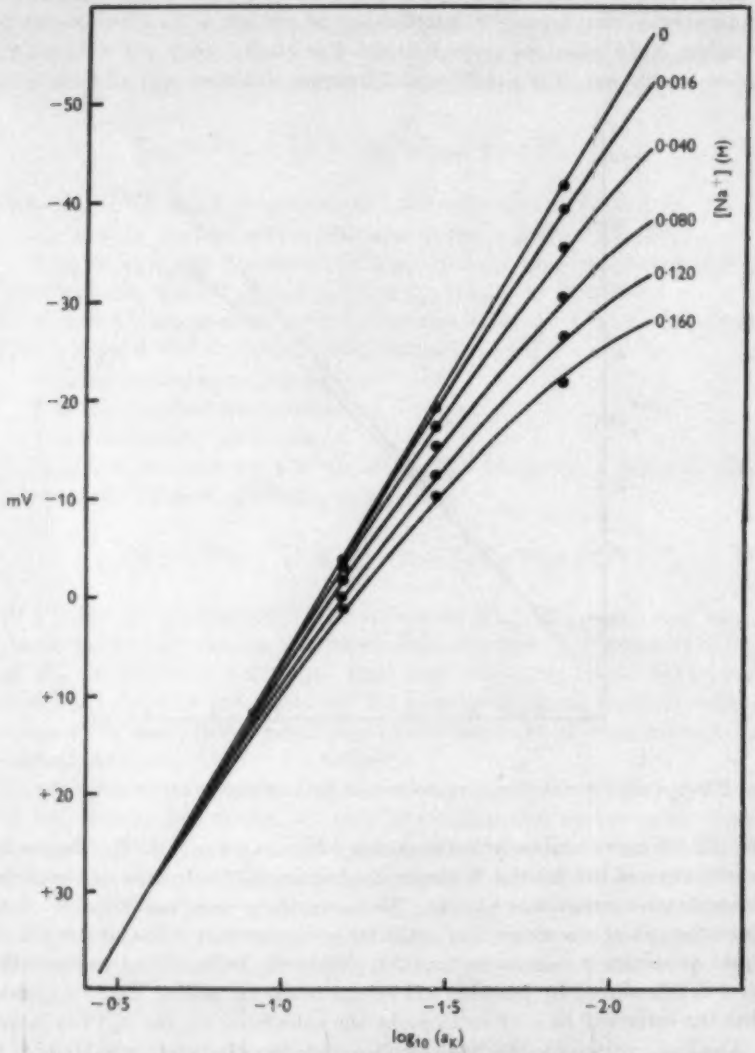

Fig. 4. Response of a K micro-electrode to K^+ in mixtures of NaCl and KCl. The points are experimental and the curves were drawn according to eqn. 4 determined from the standards, 0·01 M and 0·10 M-KCl, and 0·10 M-NaCl.

0·10 M-KCl and 0·10 M-NaCl, it was important to check how well this equation predicted the response in KCl-NaCl mixtures. The response of a K electrode ($k_{KNa} = 0{\cdot}10$) in known mixtures is illustrated in Fig. 4. The points are experimental and the curves are drawn according to the equation determined from the three standards. Note that the curves pass through the experimental points except when $[Na^+]$ is very large compared to $[K^+]$.

TABLE 3. Response of sodium glass micro-electrode in serial dilutions of 0·55 M-NaCl (pH 8·0)

Soln. no.	Concentration of Na^+ by flame photometry (M)	$\gamma\pm$*	$\bar{a}_{Na}$	Sodium glass electrode	
				E_{Na} (mV)	a_{Na}
1	0·540	0·678	0·366	96·5	0·385
2	0·360	0·700	0·252	87·0	0·265
3	0·195	0·735	0·143	72·5	0·150
4	0·106	0·781	0·0826	57·5	0·083
5	0·101	0·778	(0·078)Δ	(56·0)Δ	0·078
6	0·0425	0·848	0·036	36·0	0·036
7	0·0095	0·905	(0·0086)Δ	(1·5)Δ	0·0086

* Mean activity coefficients (25° C), from Robinson & Stokes (1959). Δ Formula for electrode determined from $\bar{a}_{Na}$ and E_{Na} of solutions 5 and 7.

The Na electrode had to be checked in Na concentrations up to 0·5 M if any reliance was to be placed on the electrode's response in sea water. Serial dilutions of a 0·55 M-NaCl stock solution were buffered to pH 8·0, tested with the Na electrode, then diluted and analysed for Na by an EEL flame photometer. Table 3 lists the results. The concentration values from flame photometry are converted to mean activities ($\bar{a}_{Na}$) so that they compare with the Na electrode activities (a_{Na}). The formula for the Na electrode was determined from $\bar{a}_{Na}$ and E_{Na} of solutions 5 and 7. The other values of $\bar{a}_{Na}$ and a_{Na} agree well except at high concentrations. This deviation may be a combination of the following errors: (*a*) E_{Na} was only measured to the nearest 0·5 mV, which may not be precise enough for high concentrations; (*b*) E_{Na} is increased by small increases in junction potential at the reference electrode as concentration increases; (*c*) some error in flame photometry values may occur as a result of diluting. The same deviation was noted in serial dilutions of sea water and artificial sea water. Therefore, the Na electrode value of a_{Na} in sea water must be regarded as 5–10% too large.

Similar results to those in Table 3 were noted with the K electrode in serial dilutions of 0·5 M-KCl, except that the deviations from the expected potassium activity ($\bar{a}_K$) at the higher concentrations were smaller. Therefore, this factor was regarded as relatively unimportant considering the

limited reliability of the K electrode in detecting K^+ in the presence of Na^+, its relative selectivity being small (2·5 to 10 times) compared to that of the Na electrode (200 times).

TABLE 4. Electrode measurements on axons

Temperature range for all axons, 17–19° C. Experiments in Table 4*a* were done in November, December and January 1959–60, and those in Table 4*b* were done in February and March 1960. Axons 9, 10, 14, 19 and 22 were stored in sea water at 0° C for 15–20 hr before they were cannulated and tested.

Axon. no.	Na electrode 'outside-inside' (mV)	Amplitude of action potential (mV)	Resting membrane potential (mV)	Membrane reversal potential (mV)	$(a_{Na})_o$	$(a_{Na})_i$	$58\log\frac{(a_{Na})_o}{(a_{Na})_i}$	$(a_K)_i$
a, in sea water								
1	104	104	−64	40	0·361	0·040	55·5	0·223
2	103	100	−55	45	0·300	0·0285	59·0	0·205
3	116	116	−58	58	0·346	0·0323	60·0	0·218
4	115	116	−60	56	0·346	0·0351	58·0	0·201
5	117	108	−58	50	0·366	0·0361	58·0	0·211
6	118	108	−62	46	0·366	0·0390	56·0	0·215
7	124	120	−66	54	0·366	0·0361	58·0	0·199
8	120	107	−58	49	0·366	0·0323	61·0	0·194
9	111	109	−55	54	0·370	0·0437	54·0	0·168
10	110	108	−59	49	0·370	0·0494	51·0	0·176
11	116	105	−55	50	0·352	0·0285	63·0	0·212
12	128	116	−59	57	0·361	0·0304	62·5	0·228
13	118	109	−59	50	0·361	0·0456	52·0	0·196
14	113	104	−59	45	0·356	0·0465	51·0	0·200
Average	115·0	109·3	−59·1	50·2	0·356	0·0374	57·0	0·203
S.E. of mean						±0·0019		±0·0046
b, in artificial sea water								
15	130	110	−60	50	0·380	0·0294	64·5	0·187
16	115	104	−54	50	0·380	0·0332	61·5	0·175
17	129	108	−56	52	0·380	0·0218	72·0	0·148
18	126	108	−57	51	0·380	0·0275	66·0	0·177
19	124	104	−50	54	0·370	0·0256	67·5	0·185
20	116	100	−55	45	0·370	0·0361	58·5	0·177
21	136	103	−61	42	0·375	0·0209	73·0	0·172
22	130	103	−61	42	0·378	0·0285	65·0	0·154
23	126	100	−55	45	0·370	0·0266	66·0	0·159
Average	126	104·5	−56·5	48·0	0·376	0·0275	66·0	0·170
S.E. of mean						±0·0017		±0·0047

Na and K activities in axons

Table 4*a* and 4*b* lists the results obtained at 20 mm depth in axons at 17–19°C bathed in sea water and artificial sea water. Axons were included only if: (*a*) the amplitude of the action potential was greater than 100 mV; (*b*) both a_{Na} and a_K were measured; (*c*) the resting membrane and action potentials were the same before and after the four insertions. Measurements were usually made 30–60 min after the axon was cannulated and mounted in the recording cell.

The first column of Table 4 is the difference between the Na glass electrode readings outside and inside the axon. This difference includes the resting membrane potential and 'Na gradient potential' across the membrane. According to Hodgkin & Huxley (1952*a*) and Hodgkin (1957), the amplitude of the action potential is made up of a collapse of the resting membrane potential plus a reversal potential which approaches the Na gradient potential. If this theory is correct, the difference between external and internal readings with the Na electrode must be equal to, or greater than, the action potential. Fibre no. 4 was the only axon which gave a higher action potential and this only by 1 mV. None of the axons excluded from this table gave an action potential greater than the difference between the outside and inside Na electrode potentials.

The same relationship exists, of course, between the calculated Na gradient potential and the reversal potential. $(a_{Na})_o$ and $(a_{Na})_i$, the external and internal sodium activities respectively, were calculated from eqn. (5), for which the constants were derived before and after each experiment from standards. The membrane potential was subtracted from the internal voltage reading before $(a_{Na})_i$ was calculated, and added to the action potential to get the reversal potential. Junction potential was ignored for this comparison, since it affects the reversal potential and $(a_{Na})_i$ in the same way. $(a_K)_o$ was not calculated, for reasons already given.

The absolute values of $(a_{Na})_o$ are subject to an error which was noted in Table 3. The approximate Na content of sea water (Table 2) was 0·47 M and if the mean activity coefficient is taken to be equivalent to that for 0·47 M-NaCl (0·685), then the expected mean activity ($\bar{a}_{Na}$) is 0·32 compared to the observed value of 0·356 (Table 4*a*). If the former value is accepted as more nearly correct, then the experimentally obtained $(a_{Na})_o$ must be regarded as 5–10 % too large. This error in estimating the absolute values of a_{Na} should have no effect on the comparison between the action potential and the difference between the potential of the sodium electrode outside and inside the axon.

The axons bathed in artificial sea water (Table 4*b*) gave average resting and action potentials a few millivolts less than axons in sea water, and $(a_{Na})_i$ as well as $(a_K)_i$ was consistently lower than in Table 4*a*. No adequate explanation was found for these discrepancies.

Variation of $(a_{Na})_i$ and $(a_K)_i$ with depth below tip of cannula

In some experiments readings were taken near 0 and 10 mm as well as at 20 mm depth from the cannula. Table 5 shows readings from 27 axons in sea water, grouped according to their depth. Only one set of measurements is included from an axon at a given depth, taken 30–60 min after cannulation. With a few exceptions, an axon contributed either three sets of

measurements (0, 10, 20 mm depth) or two sets of measurements (10, 20 mm depth). Concurrent with the sharp drop in resting and action potential noted as the electrodes approached the cannula, there was an increase in $(a_{Na})_i$ and decrease in $(a_K)_i$. The average changes between 20 and 10 mm depth were not large since the 10–15 mm group of Table 5 contained many measurements similar to those in the 15–20 mm group. The last column shows that the sum of the free Na and K contents remained constant in spite of the changes in composition.

TABLE 5. Average Na and K activities of 27 axons in sea water at varying depths from cannula (17–19° C)

No. of axons	Depth from cannula (mm)	Resting potential ±S.E. (mV)	Action potential ±S.E. (mV)	$(a_{Na})_i$ ±S.E.	$(a_K)_i$ ±S.E.	$(a_{Na})_i + (a_K)_i$
8	0–5	36·6 ± 4·0	48·8 ± 9·4	0·0790 ± 0·003	0·1500 ± 0·015	0·2290
25	10–15	55·4 ± 1·0	100·0 ± 2·0	0·0455 ± 0·001	0·1875 ± 0·004	0·2330
13	15–20	57·7 ± 1·1	111·0 ± 1·7	0·0332 ± 0·0008	0·2045 ± 0·004	0·2377

Analysis of measurements on all axons in sea water

In Table 4*a* axons with an action potential higher than 100 mV were selected, and in Table 5 axons were grouped according to the depth at which measurements were taken. It was considered worth while to analyse all the results regardless of depth from cannula or size of the action potential (Figs. 5 and 6). Again, only one set of measurements at a given depth and 30–60 min after cannulation was taken from each axon. In Fig. 5 the Na gradient potential (V_{Na}) was plotted against the reversal potential ($AP+RP$), and in Fig. 6 the K gradient potential (V_K) was plotted against the resting potential. Since $[K^+] = 0{\cdot}0105$ M in sea water, and $[Na^+] = 0{\cdot}470$ M, and the activity coefficient of Na was $0{\cdot}356/0{\cdot}470 = 0{\cdot}760$ (Table 4*a*), $(a_K)_o$ was taken as 0·008 for all readings of $(a_K)_i$. It was possible to represent the results in four groups, which are shown as rectangles. The sides of each rectangle are equal to twice the standard error around the mean value for that group. The number of experiments is indicated beside each rectangle. The straight line corresponds to equality between ordinates and abscissae.

Figure 5 illustrates how the reversal potential approached V_{Na} closer the higher the action potential and the lower the internal Na content. Conversely, as measurements were made nearer the cannula, the reversal potential fell more rapidly than V_{Na}. Similarly, the resting potential fell more than V_K as one approached the cannula (Fig. 6). This rapid falling away of resting and reversal potential as the cannula is approached can be explained by the effect of increased leakage of the transmembrane potential as the measurements are taken nearer to the cut end of the fibre.

However, it was noted in most experiments that the reversal potential dropped faster than the resting potential, as can be seen from the average figures in Table 5. From 20 to 0 mm the resting potential fell 21 mV, compared to 41 mV for the reversal potential. Similarly, in Figs. 6 and 5, the resting and reversal potential dropped 32 and 50·5 mV, respectively.

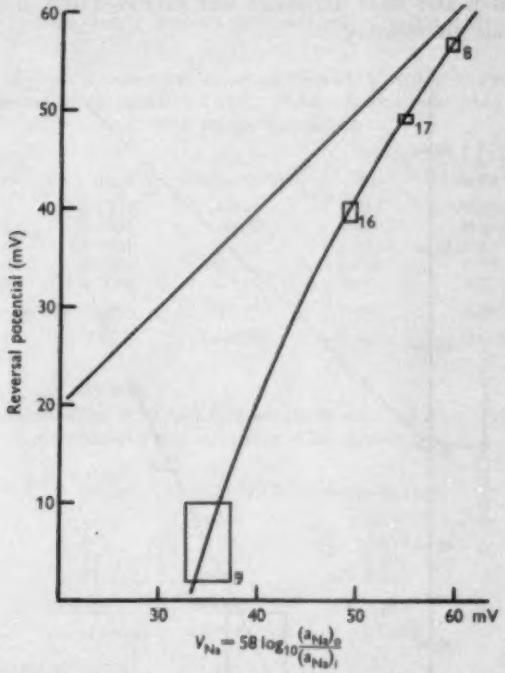

Fig. 5. Mean membrane reversal potential versus mean Na equilibrium potential of four groups of axons in sea water (17–19° C). The straight line corresponds to equality between ordinates and abscissae. The sides of a rectangle equal twice the standard error for each group. The number of experiments is indicated beside each rectangle.

The disproportionate reduction in reversal potential is presumably due to inactivation of the Na entry in the active state brought about by the lowering of the resting potential (Hodgkin & Huxley, 1952*b*).

Even at 15–20 mm depth where the effects of short-circuiting are minimal V_K was much larger than ther esting potential. For example, at the top rectangle 6 (Fig. 6), V_K exceeded the resting potential by 20·5 mV, when the corresponding rectangle 8 (Fig. 5) showed V_{Na} only 3 mV greater than the reversal potential. Hodgkin & Katz (1949*a*) noted a similar

difference, and suggested that the resting membrane might be permeable to Cl^- and Na^+ ions as well as K^+ ions, but to a lesser degree.

Na and K analysis on extruded axoplasm

Some axons were removed from the sea-water bath after measurements were completed, and their axoplasm was extruded and analysed for Na and K by flame photometry.

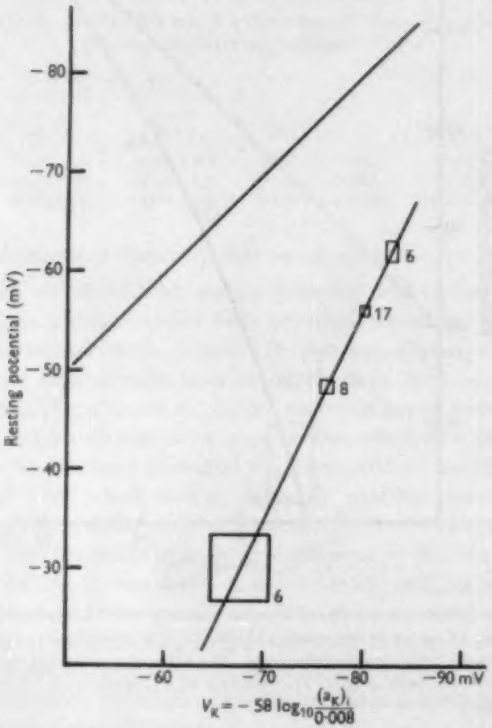

Fig. 6. Mean resting potential versus mean K equilibrium potential of four groups of axons in sea water (17–19° C). See also legend to Fig. 5.

The technique consisted in blotting the axon, and laying it on a roll of dry filter paper, except for one cut end which rested on a small weighed square of polyethylene film. The axoplasm was extruded on to the film by running a small roller along the axon. The sample was placed in a weighed Pyrex bottle and reweighed. Distilled water was added and the specimen allowed to stand for at least 24 hr before flame analysis. A couple of drops of 1 M-HNO_3 and 2 M-$CaCl_2$ were added to the distilled water to help dissolve the axoplasm (Hodgkin & Katz, 1949*b*; Chambers and Kao, 1952).

The results from this method are shown in Table 6. Also included are the $(a_{Na})_i$ and $(a_K)_i$ values before the axon was removed from the recording cell. The axoplasm was assumed to have a water content of 88 % by weight (Manery, 1939). Compared with the results by other investigators (Steinbach & Spiegelman, 1943; Keynes & Lewis, 1951; Koechlin, 1955), the $[K^+]$ values are acceptable but the $[Na^+]$ values are too large. These large $[Na^+]$ values are probably due to contamination from extracellular fluid.

TABLE 6. Na and K concentrations in axoplasm extruded after activities had been measured in the cannulated axon. Concentrations were measured on an EEL Flame Photometer

Axon no.	a_{Na}	$[Na^+]$ (mole/kg H_2O)	a_K	$[K^+]$ (mole/kg H_2O)
3	0·0323	0·144	0·218	0·359
4	0·0351	0·157	0·201	0·323
6	0·0390	0·136	0·215	0·403
7	0·0361	0·153	0·199	0·387
43	0·0408	0·146	—	0·376
Average	0·0370	0·147	0·208	0·369
S.E. of mean	±0·0017	±0·0036	±0·0048	±0·013

TABLE 7. Concentration of Na and K in axoplasm extruded from freshly dissected axons after 2 min of soaking in iso-osmotic glucose

Axon no.	$[Na^+]$ (mole/kg H_2O)	$[K^+]$ (mole/kg H_2O)
44	0·097	0·386
45	0·101	0·412
46	0·063	0·400
47	0·078	0·413
Average	0·085	0·403
S.E. of mean	±0·0088	±0·0063

This was checked by changing the technique for four freshly dissected axons (no cannulation or impalements were made). The axons were thoroughly cleaned and then tested with external electrodes for transmission of an action potential throughout their length. They were soaked in iso-osmotic glucose for 2 min, blotted, and the axoplasm extruded as before. Analysis of these fibres (Table 7) gave a much lower $[Na^+]$ and a slightly higher $[K^+]$.

Even with these technical improvements, the cation concentrations would still not be strictly comparable to the activity measurements in the mounted axon. It was therefore considered more desirable to extrude the axoplasm first, and then measure the cation activities before diluting the axoplasm for flame analysis. In this way any changes in the axoplasm would affect both activity and concentration values.

Several Pyrex cannulae were drawn similar to the mounting cannula in Fig. 2, but with tips 400 and 500 μ in diameter and capillaries about 2 cm long. These were placed in polystyrene bottles and weighed. Each fibre was cleaned and tested with external electrodes for transmission of an action potential throughout its length. The fibre was soaked in isosmotic glucose for 2 min, then placed on the glass stage of a binocular microscope and cannulated. The cannula was ligated firmly in position with two silk ties. The axoplasm was extruded into the cannula and the empty axon removed. The filled cannula was mounted in the recording chamber (containing 0·58 M-KCl) so that the tip was just below the bath solution. Readings from an open-tipped micro-electrode were made inside and outside the cannula to detect any potential difference across the cannula tip. Not more than $\pm$ 2 mV was recorded. Na and K electrode potentials were taken in the middle of the column of axoplasm. The cannula was dismounted, wiped dry and placed in the appropriate bottle for re-weighing and diluting for flame analysis. About 3 min were required for cannulating, ligating and mounting, and about 5 min for electrode readings.

TABLE 8. Measurements of sodium and potassium activity and concentration on extruded axoplasm (17–19° C)

Axon no.	a_{Na}	[Na^+] (mole/kg H_2O)	a_K	[K^+] (mole/kg H_2O)
33	0·0418	0·091	0·214	0·370
34	0·056	0·100	0·216	0·340
35	0·0427	0·084	0·228	0·350
36	0·038	0·125	0·191	0·343
37	0·038	0·087	—	0·315
38	0·040	0·079	—	0·348
39	0·0437	0·085	—	0·413
40	0·038	0·090	0·239	0·530
41	0·040	0·078	0·225	0·328
42	0·040	0·096	0·245	0·366
Average	0·0418	0·0915	0·223	0·370
S.E. of mean	$\pm$0·002	$\pm$0·004	$\pm$0·007	$\pm$0·019

The results from ten axons are shown in Table 8. The mean values of [Na^+] and [K^+] are not significantly different from those in Table 7. The average a_{Na} and a_K values are larger than the average activities obtained from the intact axon in sea water (Table 4*a*). Such a difference may be due to a genuine change in cation activity when axoplasm is extruded. On the other hand, the difference may be due to a difference in error between the two recording systems. For example, the values obtained from the intact axon may be too low because the recorded membrane potential was too small by 2·3–2·9 mV (the equivalent of the activity differences in Tables 4*a* and 8). Or it may be that junction potential errors in the first recorded system (intact axon) exceed the junction potential errors in the second system (extruded axoplasm) by 2·3–2·9 mV. This last possibility can be checked by calculating the individual junction potentials from the Henderson equation (1907) (Table 9). The limiting equivalent conductivities at 18°C were taken from Robinson & Stokes (1959) and sea water was assumed to be equivalent to 0·5 M-NaCl. The E_j error in Table 4*a* values can be shown to equal $-(E_j)_1+(E_j)_2-(E_j)_3$ and the E_j error in the ex-

truded axoplasm values is equal to $-(E_j)_5+(E_j)_4-(E_j)_3$. If the numerical values from Table 9 are substituted, the difference between these quantities becomes +2·6 mV. The calculation serves only to verify that junction potential errors could explain the differences between the activities from the intact axon and the extruded axoplasm, but does not rule out the first consideration that the difference may be real.

TABLE 9. Estimated junction potentials at electrodes, calculated from Henderson (1907)

Liquid junction	Junction potential (mV)	
Reference 3 M-KCl//0·5 M-NaCl	$(E_j)_1 = +0{\cdot}78$	Axon in sea water
Micro-electrode 0·58 M-KCl//0·5 M-NaCl	$(E_j)_2 = +4{\cdot}06$	
Micro-electrode 0·58 M-KCl//axoplasm	$(E_j)_3 =$?	
Micro-electrode 0·58 M-KCl//0·58 M-KCl	$(E_j)_4 = 0{\cdot}0$	Axoplasm in 0·58 M-KCl
Reference 3 M-KCl//0·58 M-KCl	$(E_j)_5 = -0{\cdot}67$	

The extruded axoplasm analysis provides evidence for the activity coefficient in the axoplasm. Hodgkin & Keynes (1953) estimated most of the potassium to be unbound. If one neglects any 'binding' of K^+, the activity coefficient of K^+ can be calculated as 0·223/0·370 = 0·605. Since Na^+ should have about the same coefficient, the free Na^+ becomes 0·0418/0·605 = 0·0695 mole/kg water. If this figure is correct, then 24 % of the total axoplasm Na was bound. This estimate will be too large if either $[Na^+]$ was too high or $[K^+]$ was too low. Or, if some of the 'active' Na^+ was squeezed from extracellular spaces then the 24 % estimate will be too low. However, the calculation is independent of junction potential since the relationship between a_K and a_{Na} remains constant no matter what the correction would be if $(E_j)_3$ (Table 9) was known.

Stimulation of axons in sea water

Measurements were made on some axons before and after stimulation. Table 10*a* shows results for axons bathed in sea water, and Table 10*b* for axons in artificial sea water. All measurements on a given axon were taken at the same depth (15–20 mm). Fibre diameters were accurate to $\pm 10\mu$. Rates of stimulation were 30/sec or 50/sec for 20–45 min. Calculated values of a_{Na} and a_K were converted to concentrations by using the calibration with standard solutions. For fibres in sea water, the average net rate of Na entry was 3·9 pmole/cm².impulse and the average net rate of K loss was 5·8 p mole/cm².impulse. For fibres in artificial sea water, Na entry was 3·7 and K loss was 5·4 pmole/cm².impulse. The difference between results in sea water and in artificial sea water was not statistically significant.

The potassium flux values in Table 10 are not very reliable, since they

depend on the accuracy of measuring a small change of a relatively large concentration. The voltage drop at the potassium electrode after stimulation was in the region of 1–3 mV. To be certain of such a change one had to measure both resting and K electrode potential with the Vibron meter

TABLE 10*a*. Effect of stimulation on intracellular sodium and potassium of axons in sea water

Temperature range 17–19° C. Na and K concentrations obtained from measured activities before and after stimulation, using mean activity coefficients (Robinson & Stokes, 1959). Axons 9, 10, 25, 27, 28 were stored at 0° C for 15–20 hr before they were cannulated and tested.

Axon no.	Diameter (μ)	Stimulation: Rate (shocks/sec)	Stimulation: Duration (min)	Before $[Na^+]_i$ (M)	After $[Na^+]_i$ (M)	Rate of gain of Na^+ (pmole/cm².imp.)	Before $[K^+]_i$ (M)	After $[K^+]_i$ (M)	Rate of loss of K^+ (pmole/cm².imp.)
8	850	30	30	0·039	0·049	3·9	0·275	0·252	9·0
9	850	30	30	0·052	0·063	4·3	0·237	0·225	4·7
10	500	30	45	0·060	0·086	4·0	0·249	0·216	5·1
11	720	30	30	0·034	0·046	4·0	0·308	0·297	3·7
12	900	30	30	0·036	0·046	4·1	0·333	0·316	7·1
14	1000	30	30	0·058	0·066	3·7	0·286	0·278	3·7
24	700	50	30	0·050	0·069	3·7	—	—	—
25	700	50	20	0·041	0·054	3·8	—	—	—
26	600	50	20	0·041	0·059	4·5	—	—	—
27	690	30	30	0·075	0·086	3·5	0·264	0·240	7·6
28	720	30	30	0·055	0·066	3·7	—	—	—
Average						3·9			5·8
S.E. of mean						±0·09			±0·8

TABLE 10*b*. Effect of stimulation on intracellular sodium and potassium of axons in artificial sea water

Axon no.	Diameter (μ)	Stimulation: Rate (shocks/sec)	Stimulation: Duration (min)	Before $[Na^+]_i$ (M)	After $[Na^+]_i$ (M)	Rate of gain of Na^+ (pmole/cm².imp.)	Before $[K^+]_i$ (M)	After $[K^+]_i$ (M)	Rate of loss of K^+ (pmole/cm².imp.)
20	540	50	23	0·044	0·063	3·6	0·250	0·220	5·9
21	540	50	20	0·025	0·041	3·6	0·243	0·219	5·2
22	560	50	23	0·034	0·051	3·5	0·215	0·184	6·3
30	770	50	25	0·034	0·048	3·6	0·234	0·215	4·9
31	765	50	25	0·041	0·058	4·3	0·243	0·222	5·3
32	540	50	25	0·050	0·072	3·9	0·250	0·224	4·7
Average						3·7			5·4
S.E. of mean						±0·12			±0·8

set at 10 mV full scale (12 cm). The stability of the Vibron on this scale was $\pm 100\ \mu$V in 10 hr. Probably the largest source of error was in the change of resting potential before and after stimulation. Even though it was recorded just as precisely as the K electrode potential, there was always some possibility that small changes occurred in the tip potential of the conventional micro-electrode, which did not cancel out when the

difference between inside and outside measurements was taken. The total potential change of the K electrode was reduced to some extent by the increase in Na^+ with stimulation. This effect was taken into account by inserting the post-stimulation value of $(a_{Na})_i$ in eqn. 4 when the post-stimulation $(a_K)_i$ was calculated. The relatively wide range in K^+ loss values (Table 10) no doubt reflects the inaccuracy inherent in the $[K^+]$ measurement. However, it is important to note that always a decrease in $[K^+]$ and never an increase was recorded with stimulation.

The recorded change in Na was more reliable, since it corresponded to a 7–10 mV change in electrode potential, which could be read quite accurately on the Vibron meter set at 30 mV full scale. Such a change was well beyond any small error in measuring resting potential before and after stimulation.

Some information on the time sequence of the changes was obtained from axons 8, 9, 10, 11, 12 and 14 (Fig. 7). In *A*, changes in resting and action potential, and in *B*, changes in $(a_{Na})_i$ and $(a_K)_i$ are plotted. The post-stimulation values for axon no. 10 were adjusted to those expected if duration of stimulation was 30 min instead of 45 min. The average changes for each recorded period are accompanied by their ranges of standard errors, if the range exceeded the diameter of the circle.

The resting potential dropped at a constant rate and was apparently unaffected by stimulation. The action potential was falling at the same rate as the resting potential before the test period, but fell much more rapidly during stimulation at 30/sec. There was a tendency for $(a_{Na})_i$ to decrease and $(a_K)_i$ to increase before stimulation, which suggests that adjustments of the fibre to the sea water were not complete and therefore more time should have been allowed after cannulation. Whether significant or not, these early trends were both reversed by stimulation. Measurements taken an hour after stimulation showed $(a_{Na})_i$, $(a_K)_i$ and action potential returning towards their prestimulation values.

DISCUSSION

The cation-selective micro-electrodes provided a simple method for direct measurement of intracellular sodium and potassium activities. The resulting values are subject to two qualifications. In the first place, the electrodes were standardized with known solutions of NaCl and KCl whose individual cation activities were regarded as equal to their mean activities. This assumption is not strictly correct but the error should be very small. The second and more important error results from liquid junction potentials at the 3 M-KCl reference electrode and the open-tipped 0·58 M-KCl micro-electrode (Table 9). Since estimates of the latter in axoplasm were doubtful, it was considered more useful to present uncorrected results.

However, two measurements which are independent of junction potential have been mentioned. One is the difference in potential of a sodium electrode inside and outside the axon (next paragraph) and the other the ratio of $(a_{Na})_i$ and $(a_K)_i$ (used for the estimate of bound Na).

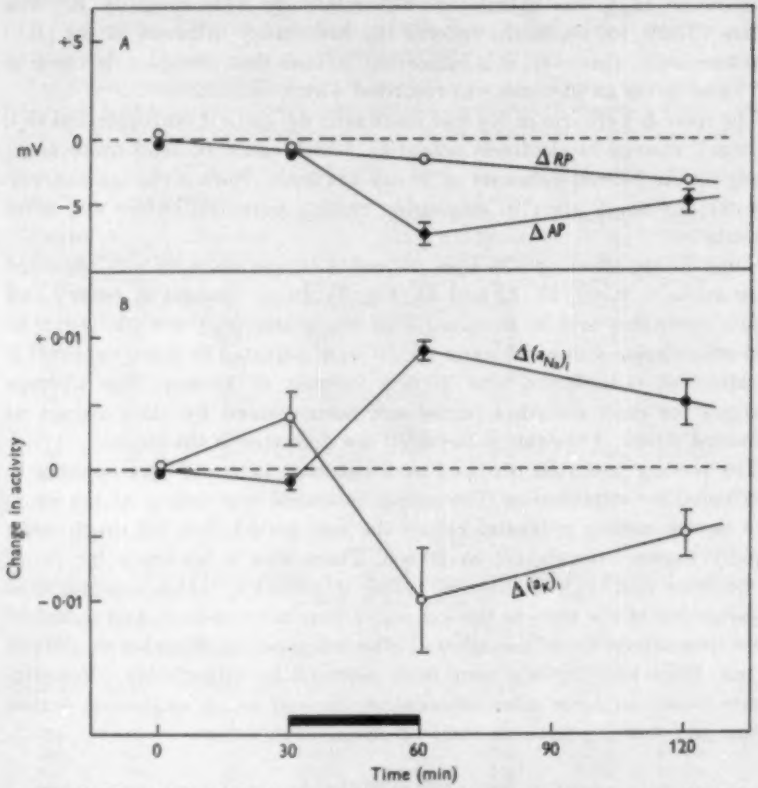

Fig. 7. Time study of the changes in $(a_{Na})_i$, $(a_K)_i$, resting and action potentials of 6 axons in sea water (17–19° C) before and after stimulation. Only the changes with respect to the initial values are plotted. The range of standard error for each recorded period is shown if it exceeds the diameter of the circle. Bar shows stimulation at 30/sec.

The outside and inside Na electrode difference is a critical test of the action potential theory put forward by Hodgkin & Huxley (1952*a*). From their voltage clamp and conductivity studies, they deduced that the membrane changed momentarily from a state in which it was mainly permeable to potassium ions to one in which it was mainly permeable to sodium ions. In other words, the amplitude of the action potential represents the sum

of the resting potential and all or part of the Na gradient potential. It follows that the measurement with a Na electrode, outside and inside, represents the maximum obtainable action potential on this theory. Only one axon (no. 4, Table 4*a*) gave an action potential in excess of this 'permissible maximum', and exceeded it only by 1 mV, which is within the limits of error of the method.

For an axon in sea water ($a_K = 0{\cdot}008$) the mean value of the internal potassium activity ($a_K = 0{\cdot}203$) leads to a potassium equilibrium potential (V_K) of 80 mV. This was about 20 mV more than the resting potential. The results therefore support the idea that the resting potential of an isolated axon in sea water is substantially less than V_K.

The cation electrodes also provided a method for measuring the net fluxes of Na and K across the membrane after a period of stimulation. The net fluxes agreed with estimates made from isotope studies (Grundfest & Nachmansohn, 1950; Rothenberg, 1950; Keynes & Lewis, 1951).

Further comment is required on the estimation of bound sodium in the axoplasm. On the assumption that the axoplasm potassium is 100 % free, the activity coefficient, γ, was 0·605 and the bound sodium 24 % of the axoplasm sodium. For the same a_K in pure KCl, Robinson & Stokes listed $\gamma \pm (25°C)$ as 0·680. Hodgkin & Keynes (1953) argued that axoplasm potassium must be at least 90 % free. Therefore, if 10 % of potassium is bound, the γ becomes 0·670 and the bound sodium increases to 31·5 %. It is tempting to think this estimate of γ is more correct, since it is closer to the value for pure KCl, but there is no evidence that the activity coefficients in axoplasm and pure KCl should be similar. In any case, not very much weight can be placed on γ since $(a_K)_i$ is uncorrected for junction potential errors. More important is the estimate of the relative degrees of binding of potassium and sodium which is quite independent of such error. Until γ can be calculated from studies on an artificial axoplasm, the present values of potassium being 100 % free and sodium 76 % free remain as reasonable provisional estimates.

SUMMARY

1. A technique is described for constructing sodium- and potassium-selective glass micro-electrodes. The electrodes were calibrated against standard solutions and inserted through a cannula into the isolated squid axon to measure intracellular sodium and potassium activities. Resting and action potential were also recorded with intracellular electrodes and at the same distance from the cannula as the cation electrodes.

2. At 20 mm depth in axons bathed in sea water (18°C), the average sodium activity was $0{\cdot}037 \pm 0{\cdot}0019$ and the average potassium activity was $0{\cdot}203 \pm 0{\cdot}0046$. In axons bathed in artificial sea water, the average

values were $0{\cdot}0275 \pm 0{\cdot}0017$ and $0{\cdot}170 \pm 0{\cdot}0047$, for sodium and potassium activities respectively.

3. The sodium activity increased and the potassium activity decreased as one approached the cannula (cut end of the axon), but the sum of sodium and potassium remained constant.

4. Sodium and potassium activities in extruded axoplasm were similar to those in isolated axons.

5. The total content of sodium and potassium in the axoplasm was determined by flame photometry. The technique was such that the concentrations could be compared directly with the activity measurements. When potassium was assumed to be 100% 'free', the axoplasm activity coefficient was calculated as 0·605. Using this estimate, the intracellular sodium was found to be only 76% 'free'.

6. Following electrical stimulation, sodium activity increased and potassium activity decreased when measured at a given distance from the cannula. The average net fluxes were:

$$\text{Na gain, } 3{\cdot}8 \pm 0{\cdot}10 \text{ pmole/cm}^2\text{.impulse;}$$
$$\text{K loss, } 5{\cdot}6 \pm 0{\cdot}80 \text{ pmole/cm}^2\text{.impulse.}$$

7. The difference between the inside and outside sodium electrode potential slightly exceeded the amplitude of the action potential. This result supports the theory that the active membrane is selectively permeable to Na ions.

8. The potassium equilibrium potential (V_K) exceeded the resting potential by about 20 mV.

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AN ELECTROPHYSIOLOGIC STUDY OF THE NEUROMUSCULAR JUNCTION IN MYASTHENIA GRAVIS

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After a study of some of the electrophysiological properties of the neuromuscular junction in isolated intercostal muscles obtained from patients with no known neuromuscular disorder (Elmqvist, Johns & Thesleff, 1960), a similar study was undertaken in patients with myasthenia gravis. The intercostal muscles were obtained by biopsy under regional anaesthesia from six patients with chronic and non-localized myasthenia gravis. Immediately upon removal the muscles were studied by the use of intracellular electrodes. Evidence will be presented for a pre-junctional deficiency of transmitter formation or transmitter release.

METHODS

The group of six patients with chronic active non-localized myasthenia gravis included one male and five females, with ages from 18 to 57 years. No patient was selected in whom there was doubt regarding the diagnosis. The study was not done on patients with acute severe myasthenia gravis at the time of the biopsy. One patient had had a thymectomy followed by a marked remission.

Intercostal muscles, usually the external, were obtained from the 5th to 7th intercostal spaces in the mid-axillary line. The muscles were carefully dissected under regional anaesthesia, and then studied under conditions similar to those described by Elmqvist *et al.* (1960). The biopsies were done on an outpatient basis, all patients tolerating the procedure extremely well, a few experiencing pain at the operative site for several hours. Five of the patients had been receiving daily doses of neostigmine or mestinon, while one was receiving no medication. In no instance was the ordinary daily medication altered during the day of the biopsy. Control muscles were obtained during thoracotomy procedures on patients of both sexes and with no known muscle disease.

The usual techniques were employed for intracellular recording with capillary glass microelectrodes (Fatt & Katz, 1951) and for iontophoretic micro-application of acetylcholine (del Castillo & Katz, 1955).

A change in the composition of the external fluid was produced either by altering the bathing fluid or by superfusing the end-plate region with solutions delivered from a pipette, as described by Nastuk (1959). The basic composition of the bathing fluid was that used by

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Liley (1956*a*). Alterations in potassium concentration were accompanied by an iso-molar change (of opposite sign) in sodium concentration.

Indirect stimulation was accomplished by stimulating fine nerve branches through a micro-pipette filled with 3M-NaCl solution. The duration of the stimulating impulse was 10 μsec.

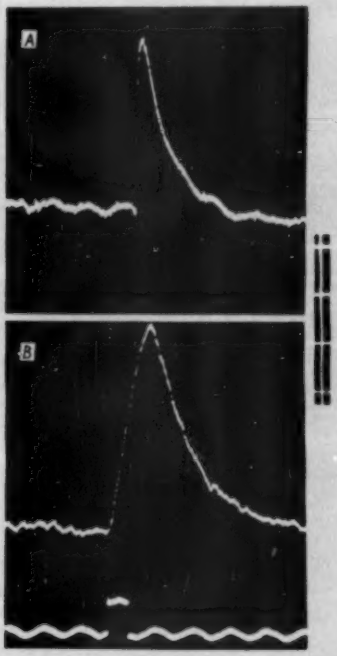

Fig. 1. The end-plate region of a single fibre from a myasthenic patient was localized (*A*) by the recording of an e.p.p. with a fast rising phase, or (*B*) by the recording of a transient membrane depolarization in response to iontophoretically applied ACh. The current through the pipette appears in the lower tracing and is equivalent to a total charge of 2×10^{-10} C. A.C. artifact 50 c/s. Voltage calibration, 1 mV.

RESULTS

The resting membrane potential of single muscle fibres was 70–90 mV, which is similar to that of normal muscle (Elmqvist *et al.* 1960). In the muscles obtained from the six myasthenic patients 97 end-plate regions were located by direct observation of fine nerve branches and either by the recording of an end-plate potential (e.p.p.) with a fast rising phase (Fig. 1 *A*), or by the recording, as shown in Fig. 1 *B*, of membrane depolarization in response to iontophoretic micro-application of acetylcholine (ACh). The total length of each of the ACh-sensitive areas was 0·1–0·2 mm.

In the great majority of the fibres from myasthenic patients spontaneous prejunctional activity, recorded as miniature end-plate potentials (m.e.p.p.s), was markedly decreased in frequency, or completely absent. In but 3 fibres from one patient were m.e.p.p.s of about normal frequency (0·2/sec) recorded (Fig. 2). In the remaining fibres only occasional m.e.p.p.s were observed (1–2/3–5 min).

The amplitude and the time course of the m.e.p.p.s were similar to those of normal muscle (Fig. 2).

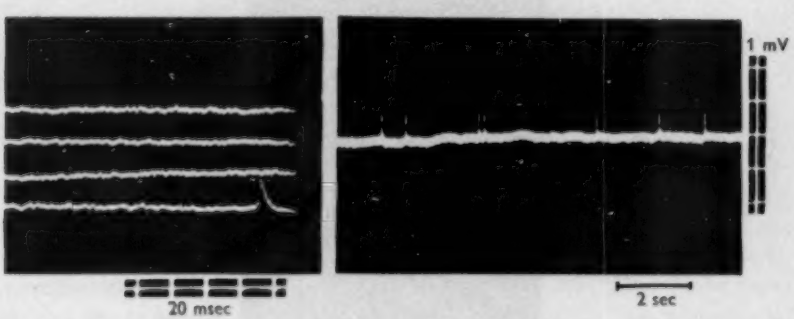

Fig. 2. Spontaneous m.e.p.p.s recorded in a muscle obtained from a patient with myasthenia gravis.

As demonstrated by Liley (1956*b*), an increase in the potassium concentration of the bathing fluid markedly increases the frequency of m.e.p.p.s. In the rat diaphragm the frequency of m.e.p.p.s increases about 1000 times in response to a 30 mM potassium solution and this change persists for at least 20 min. The amplitude of the m.e.p.p.s is reduced in proportion to the potassium-induced fall in the resting membrane potential of the muscle fibre (Liley, 1956*b*).

In normal human intercostal muscles a change in the potassium concentration from 5 to 30 mM invariably produced a long-lasting hundred- to thousandfold increase in the discharge frequency of m.e.p.p.s (Fig. 3*A*, *B*). Just as invariably, on the other hand, the application of a similar potassium concentration to muscles from myasthenic patients produced no increase in the frequency of m.e.p.p.s (Fig. 3*C*, *D*). Even after long periods of immersion (15–30 min) in solutions containing potassium in concentrations varying from 10 to 40 mM, no increase in the frequency of m.e.p.p.s was observed. The difference between normal muscles and those obtained from myasthenic patients was particularly well demonstrated when, instead of changing the bathing fluid, the neuromuscular junction was superfused by a solution containing 30 mM potassium. Immediately

following the potassium application in the normal muscle, there was a marked increase in the frequency of m.e.p.p.s, but no such change was observed in muscles from patients with myasthenia gravis (Fig. 4). An increase of the external calcium-ion concentration to twice normal did not alter the response of myasthenic muscles to potassium.

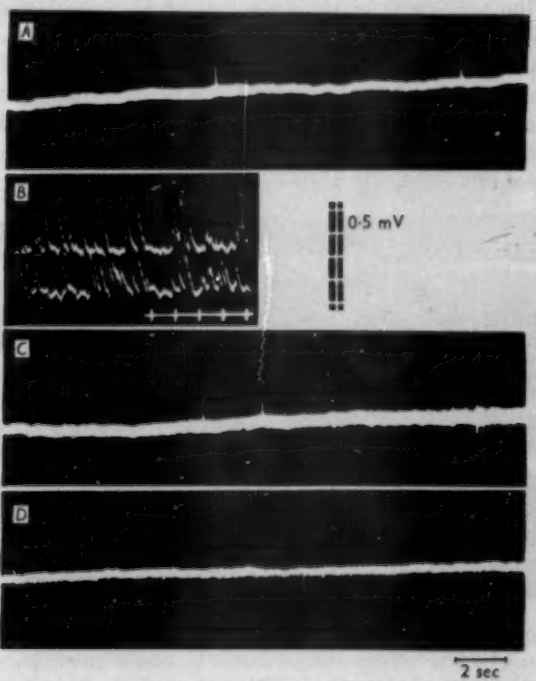

Fig. 3. The frequency of m.e.p.p.s in the presence of a solution containing 5 mM potassium (records *A*, *C*) and a solution containing 30 mM potassium (records *B*, *D*). Records *A* and *B* are from a normal muscle and *C* and *D* from a muscle of a myasthenic patient. Time marker, record *B*, 20 msec.

With potassium in concentrations of 20–30 mM the resting membrane potential of muscle fibres was reduced to 45–65 mV in both normal muscles and those from myasthenic patients. With the pipette superfusion technique the resting membrane potential was higher.

The transmitter release produced by nerve stimulation can be studied by determinations of the amplitude of e.p.p.s during and following periods of repetitive nerve stimulation. Therefore, the e.p.p. was recorded by intracellular electrodes in muscles from normal and myasthenic patients. In order to block neuromuscular transmission in normal muscles, tubo-

curarine was used (0·2–0·3 mg/100 ml.). In myasthenic muscles a short period of high-frequency nerve stimulation produced a lasting block of transmission to the majority of the fibres.

In normal muscles a brief period (2–3 sec) of repetitive nerve stimulation at a frequency of 100/sec or more, produced, after a short delay, a marked increase in the amplitude of the e.p.p., which increase lasted for about 1 min. In myasthenic muscles there was either no post-tetanic facilitation of the e.p.p., or only slight and short-lasting facilitation (Fig. 5). The amplitude of successive subthreshold e.p.p.s in a muscle from a myasthenic patient fluctuated at random without a progressive decline. In normal curarized muscle a similar stimulation frequency rapidly reduced the e.p.p. to a low and relatively stable value (Fig. 6).

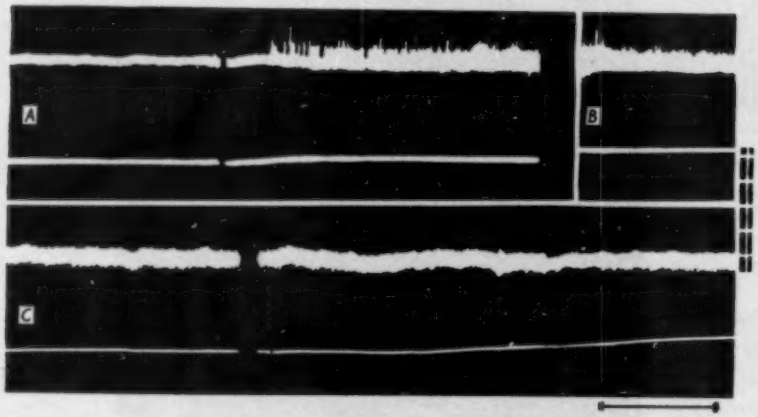

Fig. 4. When the end-plate region of a fibre is superfused from a pipette with a solution containing 30 mM potassium, a marked increase in the m.e.p.p. frequency occurs in normal muscle (*A*). Record *B* is from the same fibre after 60 min of continuous superfusion. No m.e.p.p. discharge is observed in a myasthenic muscle in response to a similar application of potassium (*C*). The m.e.p.p.s was recorded with an a.c. amplifier while the d.c. potential of the fibre is shown in the lower tracing. Onset of superfusion is marked by interruption in the tracings. Time marker, 10 sec. Voltage calibration for upper tracings: records *A* and *B*, 1 mV; record *C*, 0·5 mV; for lower tracings in all records, 10 mV.

DISCUSSION

The recording of m.e.p.p.s with normal amplitude and time course indicates that the chemical sensitivity of the post-junctional membrane in muscles from myasthenic patients is similar to that in normal healthy subjects. Furthermore, end-plate 'spots' with a high sensitivity to ACh were observed in myasthenic muscles (Fig. 1*B*). These findings make it unlikely that the neuromuscular defect is of post-junctional origin.

In muscles from patients with myasthenia gravis the low spontaneous m.e.p.p. frequency, and the lack of a frequency increase in response to potassium, suggest a pre-junctional deficiency affecting transmitter release. This possibility is supported by the absence of significant post-tetanic facilitation of the e.p.p. in such muscles. As was shown by del Castillo & Katz (1954) and Liley (1956*b*), potentiation of the e.p.p. in response to nerve stimulation involves a recruitment of transmitter

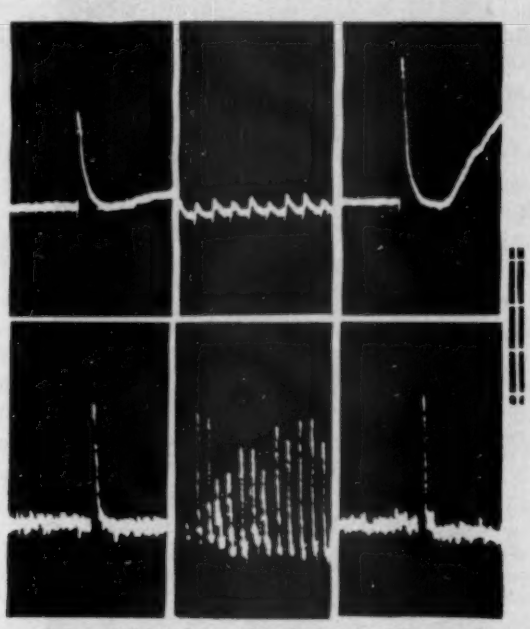

Fig. 5. E.p.p.s before (left-hand records), during (middle records) and 10 sec after (right-hand records) a period of repetitive nerve stimulation at about 100/sec. The upper records are from a curarized muscle of a normal patient and the lower ones from a non-curarized muscle of a myasthenic patient. Note that in the normal patient post-tetanic facilitation of the e.p.p. is responsible for a movement artifact. Voltage calibration: upper records, 1 mV; lower records, 0·5 mV.

release. The amplitude of successive e.p.p.s in the myasthenic preparation fluctuated at random, and depression of the response to high-frequency stimulation failed to occur when the e.p.p. was already reduced to a sub-threshold level by a preliminary period of nerve stimulation. Del Castillo & Katz (1954) demonstrated that such behaviour characterizes e.p.p.s in the presence of high magnesium- and low calcium-ion concentrations, i.e. when the quantal ACh content of transmitter release is reduced to a low value.

Thus, the results obtained from the present studies of spontaneous transmitter release, as well as of the e.p.p., strongly indicate that there exists in myasthenia gravis a deficiency affecting either transmitter formation or the transmitter release mechanism. Desmedt (1957, 1958) has reached a similar conclusion after studying post-tetanic effects with the electromyogram and isometric myogram in myasthenic patients.

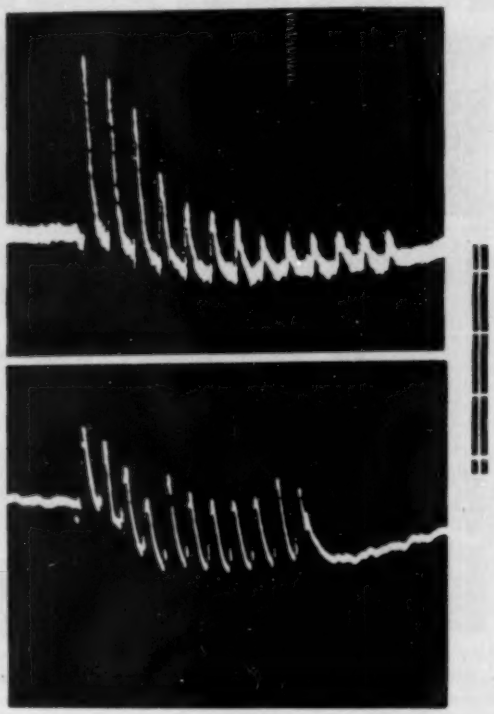

Fig. 6. E.p.p.s in response to repetitive nerve volleys at 100/sec. The upper record is from a normal curarized muscle and the lower one from a non-curarized muscle obtained from a patient with myasthenia gravis. Voltage calibration, 1 mV.

None of the myasthenic patients in our study had clinical signs or symptoms indicative of involvement of the intercostal muscles (nor of the diaphragm). Yet the pathological-physiological changes described in the present experiments were present in the intercostal muscles of *all* patients. Therefore it is likely that the disease involves all the skeletal muscles, but is clinically evident as weakness only in those most severely affected.

SUMMARY

1. Some of the electrophysiological properties of the neuromuscular junction have been studied by the use of intracellular electrodes in isolated intercostal muscles obtained from patients with myasthenia gravis and from those with no known neuromuscular disorder.

2. Spontaneous pre-junctional activity, recorded as miniature end-plate potentials (m.e.p.p.s) was markedly decreased in frequency in muscles from myasthenic patients. The application of a solution containing 10–40 mM potassium produced no increase in the frequency of m.e.p.p.s in myasthenic muscles, whereas in normal muscle it invariably caused a hundred- to thousandfold increase in the discharge frequency of m.e.p.p.s.

3. In normal muscle a short period of high-frequency nerve stimulation produced a marked post-tetanic facilitation of the amplitude of the end-plate potential (e.p.p.). In myasthenic muscle no post-tetanic facilitation, or only slight and short-lasting facilitation, was observed. During tetanic nerve stimulation the amplitude of successive subthreshold e.p.p.s in myasthenic muscles fluctuated at random without a progressive decline. In normal curarized muscle a similar stimulation frequency rapidly reduced the e.p.p. to a low and stable value.

4. It was concluded that the results obtained from the present studies of spontaneous transmitter release and of the e.p.p. strongly indicate that there exists in myasthenia gravis a prejunctional deficiency affecting either transmitter formation or the transmitter release mechanism.

We are greatly indebted to Professor T. Broman and the Department of Neurology, Sahlgrenska sjukhuset, Gothenburg, for their assistance in obtaining patients for this study. This investigation was aided by United States Public Health Service research grant B-2646 from the National Institute of Neurological Diseases and Blindness, by the Muscular Dystrophy Associations of America, Inc., and by the Air Research and Development Command, United States Air Force, through its European Office.

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METABOLIC FACTORS AFFECTING THE ELECTRICAL ACTIVITY OF INTESTINAL SMOOTH MUSCLE

BY J. AXELSSON AND EDITH BÜLBRING
From the Department of Pharmacology, University of Oxford

(*Received* 9 *December* 1960)

In a recent investigation it was found that the abolition of spontaneous spike discharges and the hyperpolarization, which adrenaline caused in intestinal smooth muscle, occurred simultaneously with an increase of phosphorylase activity (Axelsson, Bueding & Bülbring, 1959). The hyperpolarizing effect of adrenaline was found to be decreased or abolished when the muscle was depleted of glycogen by exposure to glucose-free medium.

This suggested that the effect of adrenaline on the electrical activity of smooth muscle was to some extent brought about by an influence on metabolic rate. It was therefore interesting to see if other conditions, in which a direct influence on metabolic rate could be expected, would affect the electrical activity in a similar way.

Two preliminary reports of such experiments have already been given (Axelsson & Bülbring, 1960*a*, *b*).

METHODS

All experiments were performed on the guinea-pig's taenia coli. Simultaneous records of membrane potential, action potentials and tension were obtained by the sucrose-gap method (Stämpfli, 1954; Burnstock & Straub, 1958).

Some modifications of the apparatus were made to improve temperature control and effect quick changes of temperature. Otherwise the apparatus was the same as that described by Burnstock (1958) and Bülbring & Burnstock (1960).

The composition of the bathing solution was (mM): Na^+ 137·47, Ca^{2+} 2·49, K^+ 5·93, Mg^{2+} 1·19, Cl^- 134·11, HCO_3^- 15·48, $H_2PO_4^-$ 1·19, glucose 11·50. This solution was saturated with a gas mixture of 97 % O_2 and 3 % CO_2. Its pH was 7·4–7·5. In those experiments in which glucose was withdrawn, the composition of the solution was otherwise unchanged; or, in a few experiments, the glucose was replaced by an equivalent amount of sucrose. In some experiments the NaCl was replaced by an equivalent amount of LiCl; the composition of the solution was otherwise unchanged. The temperature was usually 37° C; it could be changed by more than 10° C in one minute.

The metabolic inhibitors used were: Mono-iodoacetic acid (IAA) in concentrations from 10^{-6} to 10^{-5} and sodium azide (NaN_3) in the same concentrations.

The tension was recorded with a mechano-electric transducer valve (RCA 5734) mounted as described by Bülbring (1955). The initial tension of the muscle was adjusted to 0·5–1 g.

RESULTS

The effect of temperature changes on the spontaneous electrical activity of the smooth muscle and on the rate at which it can be electrically driven

A rise in temperature (from a range of 23–27° C to 33–37° C) temporarily slowed the frequency of spontaneous spike discharge, which in some experiments stopped for 1–2 min. Usually this effect was accompanied by a rise in membrane potential. This response to raising the temperature was seen best when the preparation had been kept from the beginning of the experiment slightly above room temperature (23–27° C) and showed relatively slow spontaneous activity.

Figure 1 shows a typical temperature effect on a muscle at very low initial tension. The rise in temperature caused immediate cessation of electrical activity, and hyperpolarization. When spike activity started again at the higher temperature it was much faster than before.

In Fig. 2 a typical effect of lowering the temperature is seen. On cooling, the activity became faster and there was a considerable depolarization. It should be noted that at 33° C the membrane potential between spikes was fairly stable. As soon as cooling started each spike was followed by a hyperpolarization and, as the frequency increased, there was a slow membrane depolarization between spikes, so that at 27° C the potential oscillated and spikes occurred on top of each wave. As the temperature declined further the frequency of discharge diminished accordingly. Later, when the temperature was allowed to reach the initial height, the membrane potential rose again and it also became stable between spikes.

When the initial tension was very high, as in Fig. 3, only a very short-lasting inhibition was seen when the temperature was raised; one spike dropped out. The higher temperature caused no secondary increase in frequency. However, each individual tension response was stronger, and the rate of relaxation became faster.

A transient inhibition during warming, varying in degree, was seen in every experiment, each including a number of observations. In some experiments the muscle was subjected to electrical stimulation. It was found to be more easily driven when moderately cooled. Conversely, warming always caused some spikes to fall out and sometimes made the preparation inexcitable for a short time.

It should be remembered that a change in the effective driving rate might be the consequence not only of a change in excitability but equally of a change in conductivity. In using the sucrose-gap method the failure to record a spike in response to a supra-maximal stimulus might be due to blocked or impaired conduction in the piece of muscle between the stimulating and recording electrodes. This objection might therefore be

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valid for all experiments where electrical stimulation was used. Similarly, when no spontaneous activity was recorded, it would only indicate that no spontaneous firing was taking place at the point of recording, but block of conduction might obscure activity elsewhere.

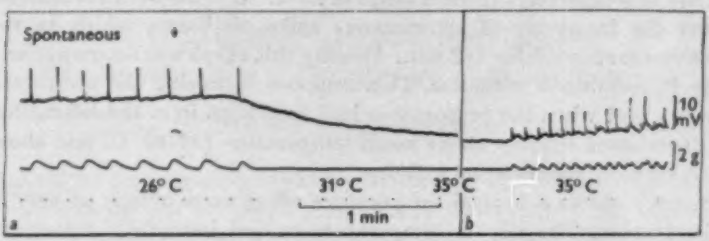

Fig. 1. Guinea-pig taenia coli. Upper record, membrane potential and electrical activity; lower record, tension. (*a*) A rise in temperature (starting at arrow) from 26 to 35° C stopped spontaneous activity and increased the membrane potential: (*b*) 2 min later spontaneous discharge returns at a faster rate in spite of the higher membrane potential.

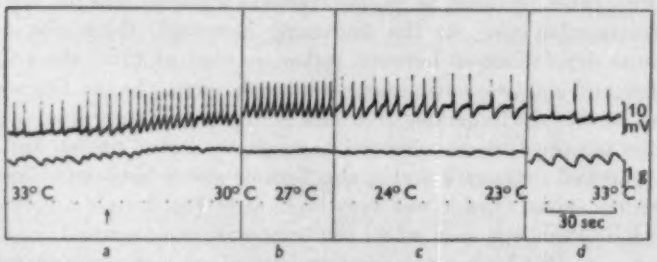

Fig. 2. The effect of cooling (starting at arrow). Records as in Fig. 1. 40 sec interval between (*a*) and (*b*); 20 sec between (*b*) and (*c*); 5 min between (*c*) and (*d*). For description see text.

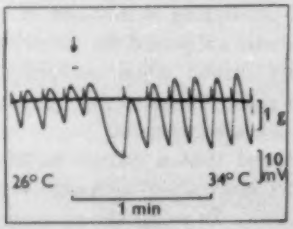

Fig. 3. The effect of warming (starting at arrow). Records as in Fig. 1.

The effect of removing and returning the external glucose

The procedure of removing and returning the external glucose could be repeated many times and at short intervals. A great number of observations could therefore be made in each experiment. Removal of glucose gradually abolished the muscle tension (Feldberg & Solandt, 1942). This was due to a failure of the spikes to evoke a tension response. Indeed, the removal of glucose from the medium usually increased the spontaneous electrical activity of the muscle. Moreover, while the range of frequencies at which the preparation could be driven in normal solution was narrow and very close to the frequency of spontaneous discharge, the driving range widened in the absence of glucose, increasing particularly towards the higher frequencies. But as the spontaneous membrane activity and its electrical excitability increased, the tension declined.

Figure 4 shows a typical effect of withdrawal of external glucose. In normal solution (*a*) the muscle responded to only every other of a series of stimuli applied at a frequency of 36/min. The spontaneous discharge was 9/min. Fifteen minutes after the removal of glucose (*b*) the muscle responded with an action potential to every stimulus (frequency, strength and duration of stimuli unchanged) and the frequency of spontaneous discharge was now 33 spikes/min. In spite of the increased rate of spike discharge the tension was now very low.

When glucose was returned to the solution a varying degree of inhibition was observed in every experiment. This inhibition was usually of long duration. The records shown in Fig. 5 are taken from the same experiment as those in Fig. 4, at a later stage after glucose had been removed repeatedly for several periods of about 15 min. The typical effect of reintroducing glucose is seen. The spontaneous spike frequency in glucose-free medium (*a*) was 24/min. The preparation could be driven up to the rate of 42/min. The frequency of 20/min was too slow, the muscle fired spontaneously between the stimuli, and there seemed to be no correlation between the stimili and the action potentials. The presence of glucose for only 4 min (*b*) slowed spontaneous activity down to 6/min and the driving rate was also greatly reduced. At a frequency of 32/min only every second or third stimulus evoked a spike, but the tension was already greatly improved. After 18 min in glucose-containing solution (*c*) the preparation could still only be driven at 3/min. After 30 min in glucose-containing solution (*d*) the maximal driving rate had risen to 20/min.

In many experiments the effect of reintroducing the glucose was best seen in the beginning, after the first long withdrawal of the external glucose. Sometimes after repeated withdrawal of glucose the effect of reintroducing it was small and short-lasting as far as the spontaneous

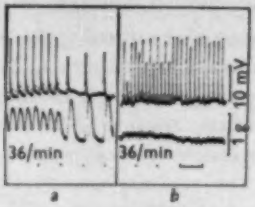

Fig. 4. The effect of removing the glucose from the bathing solution: *a* normal; *b* glucose-free. Records as in Fig. 1. Note the increase in driving rate and in spontaneous activity after the removal of glucose. Time marker, 10 sec. Further description see text.

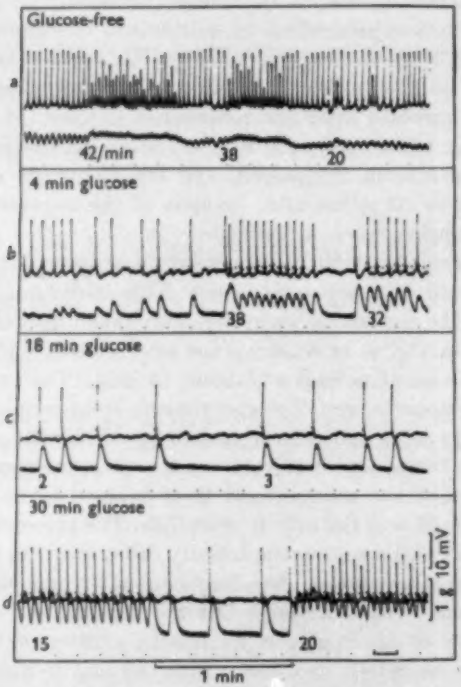

Fig. 5. The effect of returning the glucose after exposure to glucose-free solution. Records as in Fig. 1. Figures give driving rate per minute. Time marker, 10 sec. For description see text.

activity was concerned, but it was more clearly seen on the driving rate. Figure 6 shows an example of this kind. The upper record (*a*) was taken from a muscle in glucose-free solution easily driven at a frequency of 34/min. After the reintroduction of glucose the preparation soon responded with a spike only to every other stimulus. It should be noted, however, that there was at first a tension response to each stimulus. This observation indicated that the failure to record a spike was here due to a

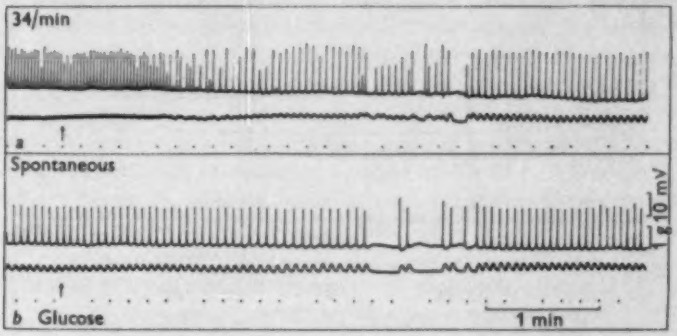

Fig. 6. The effect of returning glucose (at arrow) after repeated exposure to glucose-free medium, totalling 45 min in (*a*) and 85 min in (*b*). The depressed excitability is seen in the driven preparation in (*a*), a more transient effect on spontaneous activity in (*b*).

failure in conduction. The lower record (*b*) shows the effect of the third application of glucose, after 20 min withdrawal, on the spontaneous activity of the same muscle. There was only a very short-lasting inhibition. In both records the electrical activity was slowed while the tension increased.

The effect of metabolic inhibitors

Metabolic inhibitors have been found to cause an initial stimulation (Born & Bülbring, 1955), but Bülbring & Lüllman (1957) found that iodoacetate was an exception as, in a concentration of 10^{-3}M, it caused no increase of electrical activity. We have used fifty times lower concentrations and observed that, like dinitrophenol (DNP) and azide, iodoacetate increased the rate of spontaneous spike discharge. In one experiment it was also found to increase the driving rate of the muscle from 14 to 30/min. The effect of IAA was not easily reversible and reproducible within the same experiment.

The effect of a metabolic inhibitor on the usual effect produced by temperature change

IAA, in the concentration of 5×10^{-6} g/ml., was found to abolish the usual inhibitory effect of a sudden rise in temperature on the electrical activity of the taenia coli. Figure 7 shows that the rise in temperature, which in normal conditions (*a*) stopped the spike activity, in the presence

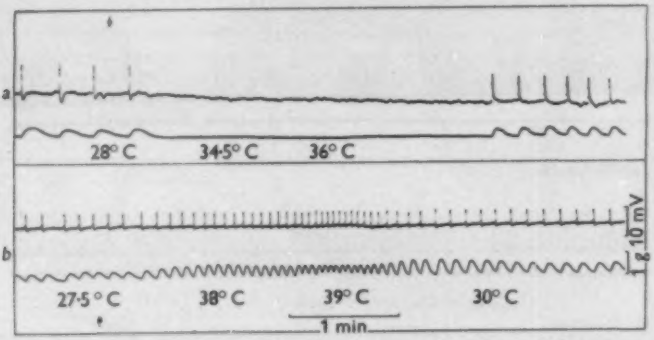

Fig. 7. The abolition of the effect of raising the temperature by iodacetate: (*a*) normal solution; (*b*) after 27 min in IAA. Note transient inhibition of electrical activity and hyperpolarization in (*a*) and immediate acceleration of spike discharge in (*b*). Records as in Fig. 1.

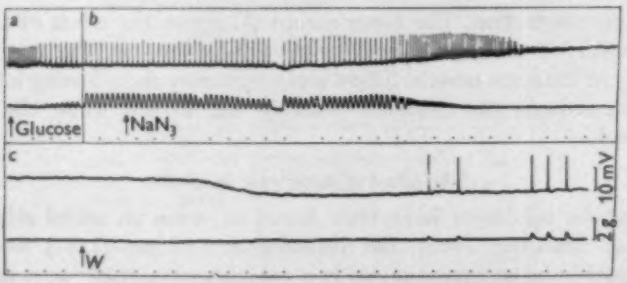

Fig. 8. The effect of returning glucose after prolonged exposure to glucose-free solution (*a*); the abolition of this effect by azide, given at arrow, (*b*); and the increase of membrane polarization (*c*) after removal of azide (*W*). Records as in Fig. 1. Time marker, 10 sec.

of IAA (*b*) only increased the spike frequency. The records also show the acceleration of spontaneous activity by IAA. When lower concentrations of IAA (2×10^{-6}) were used, its effect was sometimes reversible, but it was difficult to determine a suitable concentration. Either the muscle activity

was irreversibly blocked after a very short period of increased activity, or it was not affected at all. Thus there were only a few successful experiments in that the temperature effect could be re-tested after recovery.

The effect of metabolic inhibitors on the effect of returning the glucose after a period of depletion

Metabolic inhibitors also abolished the inhibitory action of restoring the normal glucose concentration after a period of depletion. The record in Fig. 8*a* is taken from a preparation which in the absence of glucose could be driven at a frequency of 44/min. When glucose was added (*a*) the preparation failed to respond and a spike was only evoked by every other stimulus. The addition of NaN_3 in (*b*) abolished the effect of glucose and the preparation became more excitable again. It then became depolarized to such an extent that stimulation became ineffective. When NaN_3 was washed out (*c*) at *W*, glucose being present throughout, the membrane repolarized and spike activity reappeared.

The effect of removal of glucose on changes in electrical activity normally produced by temperature changes

In these experiments the temperature effect was first recorded in normal conditions. Then the glucose was withdrawn from the solution. During the gradual depletion the effect of raising the temperature was tried repeatedly and it was found that its inhibitory effect became gradually weaker until it was fully abolished. The abolition usually coincided with the point when the tension response was nearly abolished, which occurred after a period varying from $\frac{1}{2}$ to 2 hr. The glucose was then returned to the bathing solution and, as soon as the first inhibitory effect of the glucose had passed off, the effect of raising the temperature was tested again. It then produced its usual inhibitory action.

In Fig. 9 the inhibitory action of a rise in temperature in normal solution is not shown. After 80 min in glucose-free solution (*a*) the rise in temperature had still some inhibitory effect. After 2 hr in glucose-free solution (*b*) the tension response was almost abolished. In spite of this, and in spite of the temperature being lower than in (*a*), the spontaneous activity was faster. A rise in temperature had now no inhibitory effect, it only accelerated the spike frequency.

The effect of substituting Li for Na on the usual effect of glucose and of raising the temperature

In two experiments all the NaCl of the test solution was replaced by LiCl, Na being present only as $NaHCO_3$ in its normal concentration. The

effect of removing the glucose and its replacement was tested, both on spontaneous activity and driving rate.

The results are shown in Fig. 10. In (*a*) spontaneous activity is seen first, then the spikes increased in amplitude when they were evoked by electrical stimulation (33/min). This muscle had been in glucose-free

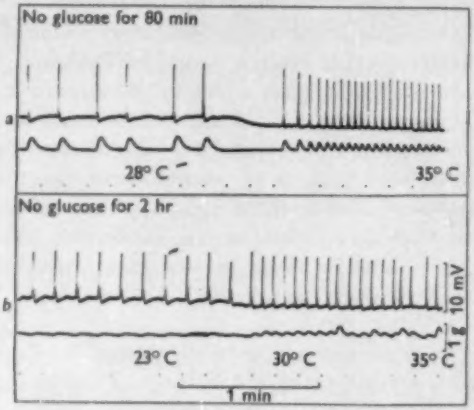

Fig. 9. The inhibitory action on raising the temperature (*a*) being gradually abolished by prolonged exposure to glucose-free solution (*b*). Records as in Fig. 1.

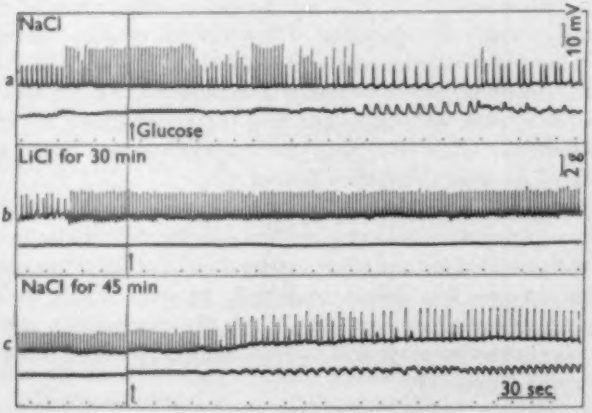

Fig. 10. In each record the muscle had been exposed to glucose-free solution for 30 min. Substitution of NaCl by LiCl (*b*) abolished the inhibition caused by returning glucose (at arrow) to the medium containing NaCl (*a*) and (*c*). In (*a*) and (*b*) a short period of spontaneous activity is shown before electrical stimulation began. In (*c*) the preparation was electrically stimulated throughout. Records as in Fig. 1.

solution for 30 min and the usual inhibitory effect of glucose was seen. In (*b*) the same procedure was repeated, but in a solution which contained Na in only 11% of the normal concentration. Glucose now had no effect. In (*c*) after the normal Na concentration had been restored glucose regained its inhibitory action.

One similar experiment was done in which the effect of raising the temperature was tested in low sodium. It was also found to be reduced in lithium solution.

DISCUSSION

A rapid increase in temperature from 23–27° C to 33–37° C was found to cause a transient depression or abolition of spontaneous electrical activity. It was also found to depress the conducted response to electrical stimulation. This observation was consistent with the hypothesis that the inhibition was somehow exerted via increased metabolic rate, which would be expected to rise when the temperature was raised by 10° C. Two attempts to test this hypothesis have been described. First, if the temperature effect was due to a change in metabolic rate, it should not be seen in the presence of metabolic inhibitors. Secondly, it would be expected to be abolished when the preparation was depleted of glycogen. It was found that in both conditions a rise in temperature had no inhibitory effect.

It was also found that moderate cooling caused a transient increase in spontaneous frequency and driving rate in many experiments. If this was due to a decreased metabolic rate, metabolic inhibitors would be expected to produce the same effect. This is what was observed.

On the basis of the same hypothesis it could be expected that removal of glucose as external energy source, and depletion of muscle glycogen, would accelerate the spontaneous spike discharge and facilitate electrical stimulation. This was observed in all experiments. Moreover, when the external source of energy was restored, an immediate inhibition of both the spontaneous spike discharge and of the driving rate was seen. This inhibition, produced by returning the glucose to the medium, was abolished by metabolic inhibitors.

The changes in electrical activity induced by the experimental conditions described here could thus all be explained as being secondary to changes in metabolic rate. This interpretation is supported by an observation shown in Fig. 8 of the following paper (Axelsson, Bueding & Bülbring, 1961) in which phosphorylase activity was increased 3–4 times when the temperature was raised from 26 to 36° C. Usually, when the preparation was warmed, we observed an increase in membrane polarization which coincided with and outlasted the cessation of electrical activity. The close correlation between the recorded polarization and spike activity indicated a true hyperpolarization.

It was known (Feldberg & Solandt, 1942) that removal of glucose abolished smooth-muscle tone. In the present work it was found that this was due, not to a cessation of electrical activity, but to the failure of the action potentials to evoke a tension response. When the external energy source was restored by returning the glucose to the medium the tension response was immediately increased. The increase in the force of the mechanical response coincided with the depression of the membrane excitability, providing further support for the assumption that the membrane stabilization was brought about by an increased rate of energy supply. It should be noted that the effect of returning the glucose could be easily reproduced in the same preparation once it was sufficiently depleted of glycogen.

The potency of several substances, as substitutes for glucose as external source of energy after glucose depletion, has been tested in a separate investigation (Axelsson, Bülbring & Krebs, unpublished). The relative abilities to reduce spontaneous activity and electrical excitability (driving rate) and to restore the tension response were used as criteria. This investigation showed that sodium acetate and sodium acetoacetate could be utilized after glucose depletion. Like glucose they temporarily suppressed electrical activity. Evidence that these substances provided energy was seen in the fact that their application also restored the tension response.

The similarity between the effects of returning the glucose and of raising the temperature is striking. It is tempting to think that the cause for the latter is essentially the same, i.e. a rise in the rate of energy supply. The effect of returning the glucose is usually of long duration. The temperature effect is always short-lasting, as the muscle accommodates to the higher rate of metabolism, and the final result is an increased frequency. This would be expected at higher temperature, rather than the initial change in the opposite direction.

The question remained how an increased rate of energy supply caused the membrane stabilization. If some of the energy were utilized for the active extrusion of sodium, it would become of interest to see what would happen in a sodium free solution. We did a few experiments in which the Na was substituted by Li, making the assumption that Li was not as readily pumped out as Na (Keynes & Swan, 1959). We found that the effects of both glucose and temperature were now much less and sometimes absent.

It is, however, likely that the relationship between the sources of energy or the metabolic rate and the excitability or conductivity of the smooth muscle is much more complex than through a direct stimulation or inhibition of sodium extrusion. Other factors such as interference with the

free Ca concentration as a consequence of increased metabolic rate, or primary changes in passive Na or K permeability, are alternative explanations. All these actions, together with changes in active Na transport, might play a role in determining the final results. The experimental facts presented here can, in view of the method, be subjected to many interpretations but, recognizing the indirectness of the evidence, we have come to the conclusion that the metabolic rate influences the electrical activity and excitability in intestinal smooth muscle.

SUMMARY

1. Isolated smooth muscle preparations from guinea-pig taenia coli were used for measurements of tension with simultaneous records of membrane potential and action potentials obtained with the sucrose-gap method.

2. Two types of experiments affecting the metabolic rate and the energy supply were carried out: (*a*) changing the temperature, (*b*) removing and returning the glucose in the bathing fluid.

3. A rapid rise of temperature caused a transient inhibition of spontaneous spike discharge and depressed electrical excitability. Moderate cooling increased the spontaneous spike frequency and facilitated electrical stimulation.

4. Similarly, withdrawal of glucose increased the rate at which a preparation could be electrically stimulated and sometimes also the frequency of spontaneous spike discharge. Returning the glucose to the medium caused a transient depression of the spontaneous electrical activity and of the driving rate.

5. In the absence of glucose the action potentials, which continued to be discharged for several hours, failed to evoke a tension response. This was restored by returning the glucose to the medium at the same time at which electrical activity was slowed.

6. The effects of both glucose and temperature were abolished by metabolic inhibitors. The effects of temperature were also abolished after prolonged exposure to glucose-free medium.

7. The inhibitory effects of both temperature and glucose on the electrical activity of the muscle were greatly diminished when Na was substituted by Li in the bathing fluid.

8. The influence of metabolic rate on the electrical activity of this smooth muscle is discussed.

One of the authors (J.A.) wishes to express his gratitude for a grant received from the Icelandic Scientific Research Fund.

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THE INHIBITORY ACTION OF ADRENALINE ON INTESTINAL SMOOTH MUSCLE IN RELATION TO ITS ACTION ON PHOSPHORYLASE ACTIVITY

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Previous work on the electrical changes produced by adrenaline on intestinal smooth muscle has been carried out on preparations from the guinea-pig taenia coli (Bülbring, 1954; Burnstock, 1958). This tissue exhibits rhythmic activity which is the basis of its tone. Adrenaline abolishes this tone because it stops the spontaneous spike discharge. It also depresses the conducted response when the tissue is stimulated electrically and causes an increase of the membrane potential. In the preceding paper (Axelsson & Bülbring, 1961) evidence has been presented indicating that the electrical activity in this tissue may be influenced by the metabolic rate. It was therefore of interest to know whether adrenaline, which is known to increase phosphorylase activity in some types of muscle, had this action also in the smooth muscle of the taenia coli. If so, its effect on electrical activity might be related to its metabolic action.

We therefore carried out experiments in which we measured the effect of adrenaline on phosphorylase activity of taenia coli and, simultaneously, recorded the electrical and mechanical changes which adrenaline produced in another piece of taenia taken from the same animal. We found a good correlation between the biochemical and biophysical changes, suggesting that the latter might be brought about as a consequence of an increased activation of phosphorylase.

Some of the results have been reported to the Physiological Society (Axelsson, Bueding & Bülbring, 1959; Axelsson & Bülbring, 1960).

METHODS

Adult guinea-pigs of both sexes were used. They were killed by stunning and bleeding. The taenia coli was removed by cutting out pieces which were, as far as possible, of equal length *in situ* (25–27 mm). Usually 8–10 pieces could be obtained from one guinea-pig. One end of each piece was fixed to one of a row of prongs at the lower end of a Perspex holder, while the other end was tied to one of a second row of prongs which could slide along the holder. The pieces were thus held parallel and, after immersion in the isolated organ

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bath, all pieces were stretched simultaneously to the same extent (120 % of the original length) by sliding the mobile row of prongs upwards.

At the same time one additional piece of taenia was prepared and inserted into the sucrose-gap apparatus for electrical recording. The method has been described in detail in previous papers (see Bülbring & Burnstock, 1960; Axelsson & Bülbring, 1961). As the preparation in the sucrose gap apparatus was bathed in continuously flowing solution, the fluid in the isolated organ bath containing the remaining pieces was also continuously changed by a similar slow inflow and kept at a constant level by overflow. The composition was the same as that described by Axelsson & Bülbring (1961). The temperature in both apparatus was kept constant at 35° C by the same warm-water circulating system.

The dissection Ringer's solution contained (mM): NaCl 150, KCl 5, $CaCl_2$ 2·5, glucose 11·5. The time required for dissection, weighing and setting up the preparations was on the average 40 min. Another 40 min was allowed for recovery in warm oxygenated Krebs's solution (see Axelsson & Bülbring, 1961) before the experiment was begun. The preparation from which the electrical records were obtained was subjected simultaneously to the same experimental procedures as those applied to the remaining pieces suspended in the isolated organ bath, from which they were removed, one after the other, at certain intervals of time for the determination of phosphorylase activity.

Phosphorylase activity of taenia coli was determined in the following manner. The previously weighed piece of taenia (15–25 mg) was blotted and placed in a container surrounded by ice, cut rapidly to a fine mince, transferred quantitatively into an all-glass micro-homogenizer surrounded by crushed ice and homogenized with 10 volumes of an ice-cold solution containing potassium citrate buffer (pH 6·7; 0·06 M) sodium fluoride (0·05 M) and potassium ethylene diamine tetraacetate (0·005 M): 0·1 ml of this homogenate was added to 0·2 ml. of the reaction mixture. After the addition of the homogenate the final concentrations of the constituents of the assay mixture were: 0·035 M-glucose-1-phosphate, 0·05 g glycogen/100 ml., 0·06 M potassium citrate buffer (pH 6·7), 0·02 M sodium fluoride and 0·005 M ethylene diamine tetraacetate. Immediately following the addition of the taenia homogenate an amount of 0·1 ml. (initial sample) was pipetted into 0·4 ml. of an ice-cold solution of trichloroacetic acid (5 g/100 ml.). The remainder of the assay mixture was incubated at 34° C for 13 min. After this period another 0·1 ml. (final sample) was added to 0·4 ml. of the trichloroacetic acid solution. This final mixture was centrifuged at 3500 *g* for 30 min at 0° C and inorganic phosphate was determined in a sample (0·2 ml.) of the supernatant solution according to the method of Fiske & Subbarow (1925) with the aid of an EEL colorimeter (filter number ORI; final volume, 1·0 ml.). The difference in the concentration of inorganic phosphate between the final and the initial samples was used to calculate the amount of phosphate liberated from glucose-1-phosphate during the period of incubation. No significant amounts of inorganic phosphate were formed under the same conditions when the addition of glycogen to the reaction mixture was omitted. Therefore it was concluded that the production of inorganic phosphate was brought about by the action of phosphorylase. This interpretation was confirmed by the determination of the increase in glycogen produced during incubation; it was found that the increase in glycogen was equimolar, in terms of glucose units, to the amount of inorganic phosphate liberated, according to the reaction, catalysed by phosphorylase:

$$x \text{ glucose-1-phosphate} + n\,G \rightarrow (n+x)G + x \text{ inorganic phosphate},$$

where G represents glucose units of glycogen.

Under the assay conditions described above, enzymic activity was proportional to the concentration of the homogenate and to the time of incubation over a period of 15 min. Phosphorylase activity was expressed in micromoles of inorganic phosphate liberated per minute by one gram of smooth muscle (wet weight).

In contrast to the phosphorylase of liver and of skeletal muscle the optimal priming concentration of glycogen for taenia coli phosphorylase was found to be extremely low.

Therefore it would appear that phosphorylase of smooth muscle might not be identical with the enzymes which catalyse the same reaction in skeletal muscle and liver.

Glycogen and glucose-1-phosphate were purified according to the procedure of Sutherland & Wosilait (1956). The stock solution of glucose-1-phosphate was prepared by adjusting the pH to 6·7 with HCl (2*N*). Glass-distilled water was used throughout.

While no attempt was made to determine the minimal concentrations of glucose-1-phosphate and of glycogen required for maximal phosphorylase activity, it was found that a molarity of 0·035 for glucose-1-phosphate, a concentration of 0·05 % for glycogen and a pH of 6·7 were optimal for enzymic activity. The composition of the reaction mixture used for the assay of phosphorylase activity of taenia coli was based on these observations, in order to ensure that enzymic activity was measured under optimal conditions. It should be noted that adenosine-5-monophosphate (AMP) in molar concentrations varying between 1×10^{-4} and 1×10^{-3} did not stimulate phosphorylase activity of taenia coli. Similarly, no activation was observed on addition of sulphydryl compounds (e.g. cysteine, glutathione).

In some experiments the glycogen concentrations of pieces of taenia coli (15–25 mg) were determined according to the method of Hassid & Abraham (1957). The precipitated glycogen was dissolved in 0·5 ml. of water, and the colorimetric determination was carried out on a mixture of a portion of 0·4 ml. and of 0·8 ml. of the anthrone reagent. The optical density was determined in the EEL colorimeter (filter number ORI).

RESULTS

The effect of adrenaline in normal solution

Adrenaline in concentrations of $1–2 \times 10^{-7}$ was found to increase phosphorylase activity in all experiments. The maximum effect was usually seen within 2 min. In the parallel strip, from which electrical records were obtained, adrenaline caused usually hyperpolarization (17 out of 20 observations), and this occurred, without exception, at the time at which the increase of phosphorylase activity in the parallel strip of tissue was observed.

Figure 1 shows a typical experiment. While adrenaline abolished the spontaneous spike activity, and caused an increase of the membrane potential of almost 20 mV, the phosphorylase activity, measured at the same time in another piece of taenia, increased rapidly to 126 %. It then declined again, as measured subsequently in further pieces, returning to the original value after 18 min exposure to adrenaline. The hyperpolarization persisted in this experiment for 10 min, but then the membrane potential also returned to the initial level, although adrenaline was still present. When it was washed out the membrane depolarized, and there followed a period during which spikes were discharged at a very high frequency; at this time phosphorylase activity in the parallel piece was low. After 15 min the initial conditions were restored. In normal conditions the phosphorylase activity of different pieces of taenia removed from the organ bath after various periods up to 2 hr did not differ by more than ± 5 %.

Table 1 gives 20 observations made in 13 different experiments of phos-

phorylase activity before and after the administration of adrenaline. They are arranged according to the time which elapsed between the setting up of the preparation and the determination of phosphorylase. It will be seen that, on the average, the phosphorylase activity was somewhat higher during the first hour than during the second and third hour. Accordingly, the percentage increase produced by adrenaline was less at first than later in the experiment. However, the mean values reached at any time in the presence of adrenaline did not differ by more than 2% between the two groups.

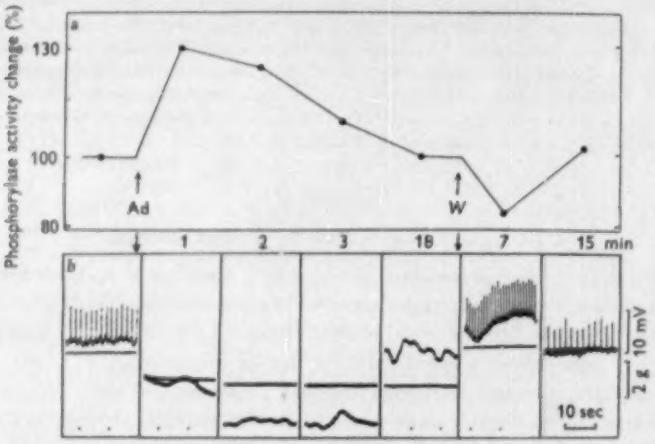

Fig. 1. The effect of adrenaline 2×10^{-7} on (*a*) phosphorylase activity and on (*b*) the electrical activity, membrane potential and tension. The records were taken at the same time as the phosphorylase activity was measured.

TABLE 1. Phosphorylase activity during the first, second and third hour after setting up the preparation, before and after adrenaline (Ad)

First hour. Phosphorylase activity (μmoleP/g)			Second and third hours. Phosphorylase activity (μmoleP/g)		
Control	After Ad	%	Control	After Ad	%
4·30	5·32	126	5·07	8·42	166
5·18	5·10	98	4·83	6·29	130
4·58	5·73	125	4·40	5·20	118
4·70	4·58	98	5·10	6·04	119
5·93	7·48	126	3·96	6·30	159
5·25	6·45	123	3·32	4·66	140
4·62	5·64	122	4·42	6·26	142
5·23	7·12	137	4·02	6·30	157
4·45	6·27	141	4·55	5·48	120
4·23	5·96	141	4·98	5·80	139
Mean 4·85	5·98	120	4·56	6·08	140

In two experiments in which adrenaline was applied repeatedly the control phosphorylase values declined in the course of the experiment ($2\frac{1}{2}$ hr) to 85 % of the first determination. In the beginning of these experiments the exceptional observations occurred, when adrenaline, although stopping the spike activity, caused depolarization. At the same time it was found to have no effect on phosphorylase activity in the parallel pieces (98 % of the controls). Later, in the course of the same experiments, when control values were lower, adrenaline increased the phosphorylase activity as it did in all other experiments.

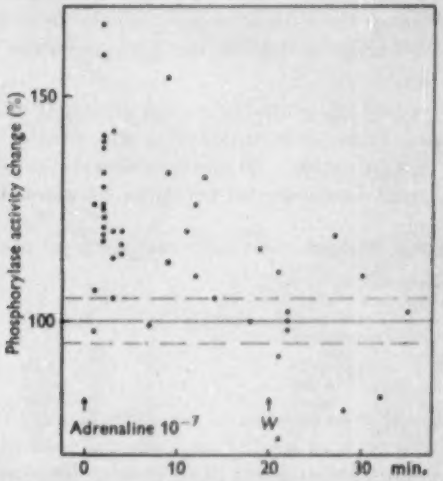

Fig. 2. The percentage change in phosphorylase activity during the exposure to adrenaline (1 and 2×10^{-7}) and after its removal (*W*).

The time course of the change in phosphorylase activity produced during an exposure to adrenaline, which lasted up to 20 min, was followed in several experiments. Figure 2 shows a diagram in which all the determinations made in the presence, and also after the removal, of adrenaline (1 or 2×10^{-7}) are presented as percentage of the first control in each experiment plotted against time. It is seen that during the exposure to adrenaline there was a steep initial rise in phosphorylase activity and the large majority of the results showed an increase above the normal range of variation (± 5 %); none were below. After the removal of adrenaline there were four observations below the normal range, four within, and three above. During this period the membrane was usually depolarized or underwent violent fluctuations. The observations were, however, too few to

allow a definite conclusion that there was a strict correlation as suggested by experiments like that shown in Fig. 1.

When lower concentrations of adrenaline were given the peak increase in phosphorylase activity was reached later. Thus, with 1 or 2×10^{-7} adrenaline the peak occurred after 2 min, with 4×10^{-8} it occurred after 7 min and with 1×10^{-8} it occurred after 12 min.

The effect of adrenaline in glucose-free solution

The results obtained with adrenaline in normal solution were consistent with the view that adrenaline might produce its biophysical effects indirectly, by increasing the rate of energy supply from glycogen breakdown. We therefore tried to deplete the glycogen store by exposure to glucose free solution.

The glycogen content of taenia coli varied greatly from one experiment to another, ranging from 0·044 to 0·128 % wet weight with a mean of 0·073 % ± 0·004 (S.E. of mean; 20 observations). Control pieces taken from the same animal, however, did not differ by more than 10 %.

TABLE 2. Glycogen content of taenia coli (% wet weight)

Hours after exposure to glucose-free medium ...	0 (30 min in normal medium)	1	2	4
Expt. 1	0·128	0·044	0·019	0·022
2	0·080	0·048	0·025	0·022
3	0·077	0·045	0·037	0·019
4	0·068	0·043	0·025	0·021

If pieces of taenia were exposed to dissecting Ringer's solution (room temperature, not oxygenated) for 3 hr they lost 50 % of their glycogen content. The glycogen loss during the average dissection period of about 40 min was not determined, but after 30 min suspension in oxygenated Krebs's solution at 37° C the normal glycogen content was present. It remained for 3 hr in normal conditions in two experiments, in another it fell to 70 % of the initial value.

Exposure to glucose-free solution led to a progressive reduction in glycogen content. The results of four experiments are shown in Table 2. Within 1 hr the glycogen content fell to 50 %, within $2\frac{1}{2}$ hr to 30 %, but after 4 hr it was still 25 % of the normal control. Thus, within the period of our experiments the removal of glucose from the medium severely diminished the glycogen store, but did not deplete it completely.

The biophysical effects produced by the removal of glucose have been described in detail in the previous paper (Axelsson & Bülbring, 1961). We found no consistent effect on the membrane potential. In four out of

seven experiments the first change after the removal of glucose was an increase in membrane potential; this was followed by depolarization. In two experiments there was little change, in one experiment we observed a progressive depolarization.

During the first stage of glucose depletion the phosphorylase activity remained either unchanged or was higher than the normal control. In this condition adrenaline had little effect or actually caused a decrease. Such a depression of phosphorylase activity by adrenaline was never seen in normal solution. The results of three experiments are shown in Table 3. During the second and third hour of exposure to glucose-free solution phosphorylase activity declined progressively and, when it had become very low, adrenaline caused a large increase bringing it back to the normal range.

TABLE 3. Phosphorylase activity (μmoleP/g) in normal and in glucose-free solution before and after adrenaline

	Normal solution		Glucose-free solution			
Expt. No.	Control	After adrenaline	Time of exposure (min)	Control	After adrenaline	Change (%)
1	—	—	18	4·53	3·65	67
			45	4·70	5·08	108
			90	5·53	6·01	109
			115	3·28	5·73	175
2	4·40	5·20	67	6·48	5·20	80
3	5·25	6·45	180	3·42	5·18	152

The biophysical effects produced by adrenaline in the absence of glucose from the medium were complex, and three aspects will have to be considered, the membrane potential, the spike activity, and the tension.

Prolonged exposure to glucose-free medium gradually abolished the hyperpolarizing action of adrenaline which, in the end, often caused depolarization instead. The gradual change is shown in Fig. 3. It may be noted that the depolarization after 4 hr exposure to glucose-free medium occurred from a very similar level of membrane potential (see reference line at beginning of each record) as the hyperpolarization produced by adrenaline after only 1 hr exposure. However, while the spikes were initially (*a*) abolished throughout the whole period of exposure to adrenaline, they returned in (*b*) and (*c*) while adrenaline was still present (not shown in the figure).

In the absence of glucose the change in the effect of adrenaline on spontaneous spike activity was indeed more consistent than the change in the effect on the membrane potential. Figures 4, 5 and 6 are all taken from the same experiment in order to show the sequence of events. In normal solution (Fig. 4*a*) adrenaline caused the usual hyperpolarization

and abolished the spikes, which remained absent throughout. After washing out (Fig. 4*b*) they returned at an increased rate and consequently the tension rose to a level higher than that seen initially. After 70 min exposure to glucose-free solution (Fig. 4*c*) the spikes remained absent for only 4 min; they can be seen to occur before adrenaline was washed out. The tension which had been low as a result of the removal of glucose was at first increased by the addition of adrenaline. When the spikes stopped (note the longer latency for the cessation to occur) the tension was of

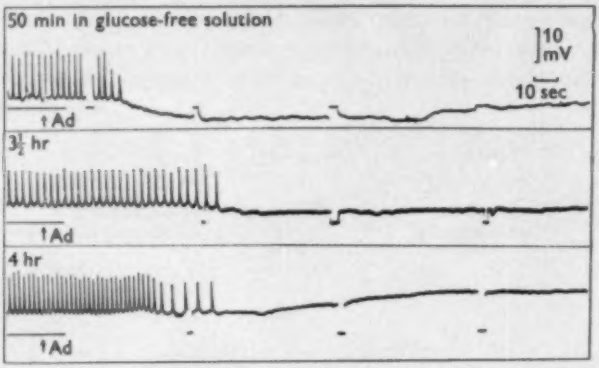

Fig. 3. The gradual change of the effect of adrenaline 10^{-7} on the membrane potential during prolonged exposure to glucose-free solution. (The records in this and subsequent figures are periodically interrupted to check possible drift.)

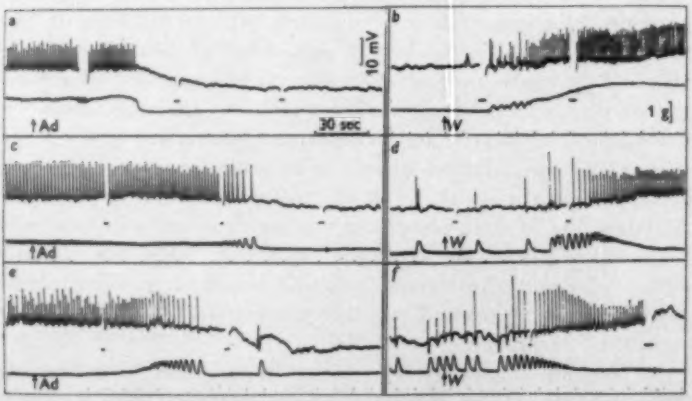

Fig. 4. The effect of adrenaline 10^{-7} on membrane potential and electrical activity (upper record) and on tension (lower record) in normal solution (*a*). After 10 min exposure, at *W*, the adrenaline was washed out (*b*). Adrenaline was given again 70 min (*c* and *d*), and 1 hr 50 min after removal of glucose (*e* and *f*). For description see text.

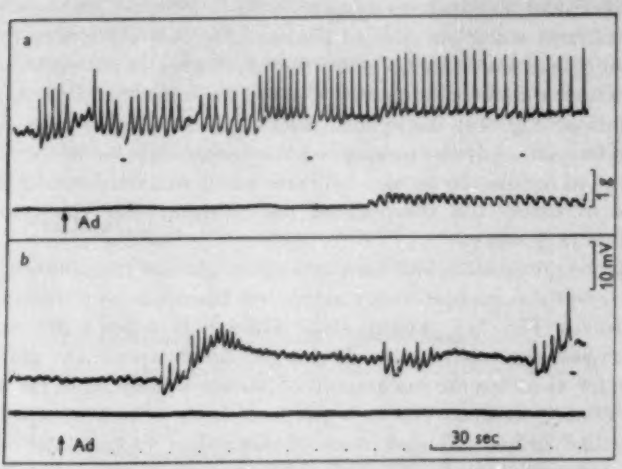

Fig. 5. Continuation of the same experiment as that shown in Fig. 4. (*a*) The effect of adrenaline in the absence of glucose for 2 hr 40 min; (*b*) after 15 min in normal solution, following 4 hr 30 min exposure to glucose-free solution.

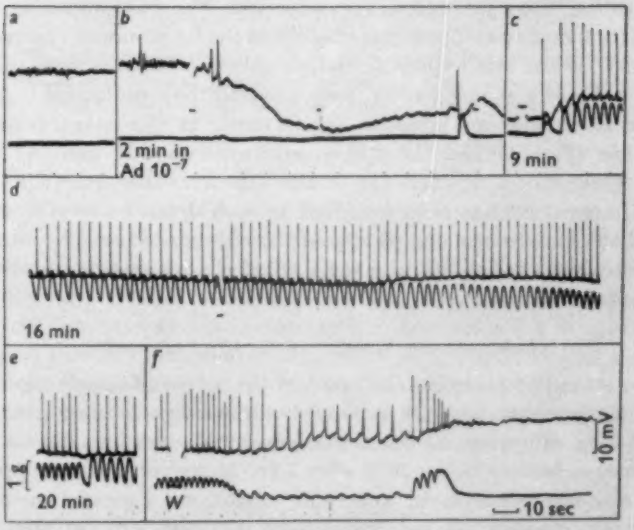

Fig. 6. Continuation of the same experiment as that of Figs. 4 and 5. The preparation had been exposed to glucose-free solution for 4 hr 30 min. Readmitting glucose for 1 hr produced no recovery (*a*). The effect of adrenaline 10^{-7} is shown in *b*, *c*, *d* and *e*, and of its removal (*W*) in *f*. For description see text.

course abolished, but as soon as spikes returned the tension response was large. After washing out the adrenaline (Fig. 4*d*) there was the usual increased rate of spike discharge but in the absence of adrenaline the tension declined and soon disappeared. After 1 hr 50 min exposure to glucose-free solution (Fig. 4*e*) the spikes, discharged at a rapid rate, failed to produce tension. Adrenaline stopped the spikes only for 90 sec. Its first effect was to restore the tension response which remained strong throughout the exposure and disappeared one minute after the removal of adrenaline (Fig. 4*f*).

When the preparation had been exposed to glucose-free solution for 2 hr 40 min it was still spontaneously active but there was no tension response to the spikes (Fig. 5*a*). At this stage adrenaline caused a depolarization and increased the rate of spike discharge. After exposure to glucose-free solution for 4½ hr the normal amount of glucose was added to the solution. This produced no recovery in a preparation in which all spontaneous activity had ceased. The addition of adrenaline 15 min later (Fig. 5*b*) caused a depolarization, and initiated spikes which produced, however, no tension. Continued exposure to normal solution failed to bring about a recovery (Fig. 6*a*). The membrane potential oscillated slightly, there was no tension. In the presence of adrenaline (given 45 min after the dose shown in Fig. 5*b*) a few spikes were observed (Fig. 6*b*), producing a trace of tension, but they stopped immediately as the membrane hyperpolarized by 20 mV. When this hyperpolarization passed off, spikes were initiated. They produced a strong tension response and they continued (*c*, *d*, *e*) as long as adrenaline was present, for 26 min. At this stage removal of adrenaline (*f*) abolished the spikes, and consequently also the tension within 2 min.

This experiment has been described in such detail because it not only showed the variety and complexity of the effects produced by adrenaline in glucose-free solution but also the gradual change from an inhibitory to a stimulant action.

Anaerobic conditions and the effect of insulin

In an attempt to increase the rate and the extent of the glycogen depletion some preparations were exposed to glucose-free solution gassed with N_2 and CO_2. However, we found that anaerobic conditions did not reduce the glycogen content below 20 % after 1 hr. Moreover, prolonged exposure to glucose-free solution in anaerobic conditions appeared to produce irreversible changes, as the tissue only regained 50 % of the original glycogen 2 hr after readmission of O_2 and the normal concentration of glucose. Increasing the glucose concentration to 5 times normal and the addition of insulin 4 u./l. had no effect on glycogen restoration. On the

other hand, if the absence of glucose and oxygen did not exceed 20 min, the tissue regained up to 80% of its original glycogen content within 60 min. In a single experiment a high glucose concentration of the medium supplemented by insulin increased glycogen resynthesis above the initial content.

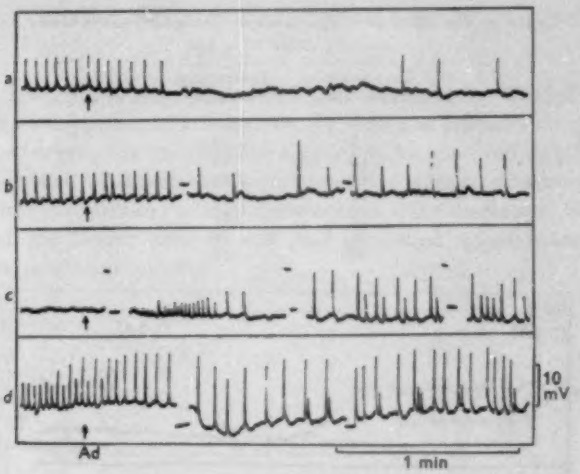

Fig. 7. The effect of adrenaline 10^{-7} on electrical activity (*a*) in normal solution; (*b*) 21 min after removal of glucose and oxygen; (*c*) 45 min after readmission of oxygen, glucose concentration 5 times normal + insulin 4 u./l.; (*d*) 15 min after (*c*).

The biophysical action of adrenaline was changed by the removal of glucose in the same way in aerobic and anaerobic conditions, i.e. the inhibitory effect was gradually abolished. In the experiment shown in Fig. 7 adrenaline caused the usual inhibition (*a*) in normal solution. After 20 min depletion (*b*) the inhibition was less. Oxygen was readmitted. Glucose in 5 times the normal concentration and insulin 4 u./l. were given at the same time. The membrane became hyperpolarized and all activity stopped (*c*). Adrenaline now led to a reappearance of spikes at a high frequency. After recovery had proceeded for 1 hr in the presence of high glucose and insulin, the usual inhibitory effect of adrenaline was slowly restored.

The effect of temperature

We also attempted to reduce the metabolic effect of adrenaline by reducing the temperature. When we measured the effect of temperature on phosphorylase activity, we found that at 20° C it was still 50% of that at

34° C, and by plotting the results shown in Table 4 it could be determined that phosphorylase activity would stop at only about 4° C.

One preparation was cooled from 37 to 5° C. At 15° C all spontaneous activity had stopped and there was a slight depolarization. At 5° C adrenaline was given, but it had no effect whatsoever on the membrane

TABLE 4. The effect of temperature on phosphorylase activity.
Activity at 34° C = 100

Temperature during assay (° C)	Percentage activity
5	8·5
10	16
15	29
17	43
19	51
21	56
23	63

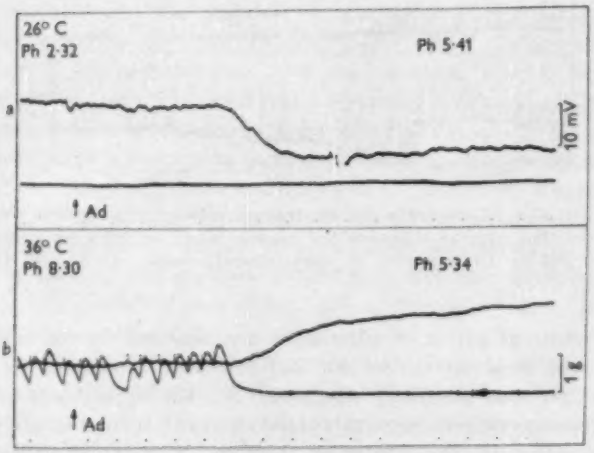

Fig. 8. The effect of adrenaline on phosphorylase activity (Ph) and on the membrane potential, (*a*) at 26° C; and (*b*) at 36° C, when the initial membrane potential was 30 mV higher than that in (*a*). Time marker 10 sec. For further description see text.

potential. Another preparation was only cooled to 30° C. At this temperature adrenaline caused slow oscillations of the membrane potential, 6 mV in amplitude at a frequency of 2–3/min.

A third preparation was set up at 26° C and kept at this temperature without an initial period at 37° C. It showed no spike activity. When adrenaline was given at this temperature it caused a large hyperpolarization, as is shown in Fig. 8*a*. At the same time it increased phosphorylase

activity from 2·32 to 5·41 μmoleP/g. Having been at 26° C for 2 hr, the preparation was warmed to 36° C. There was a 30 mV increase in the membrane potential, and after 14 min spikes appeared. Phosphorylase activity rose to 8·30 μmoleP/g (Fig. 8*b*). Adrenaline now caused depolarization though it stopped the spike activity. At the same time it reduced phosphorylase activity to 5·34 μmoleP/g.

Metabolic inhibitors

A few experiments were carried out to investigate the effect of metabolic inhibitors on the action of adrenaline. We used low concentrations to allow the effect of adrenaline on electrical activity to be observed. We found that in the presence of azide the hyperpolarizing action of adrenaline was reduced, but it continued to stop spontaneous spike discharge. When azide had acted for longer periods and had abolished spontaneous activity adrenaline produced no effect.

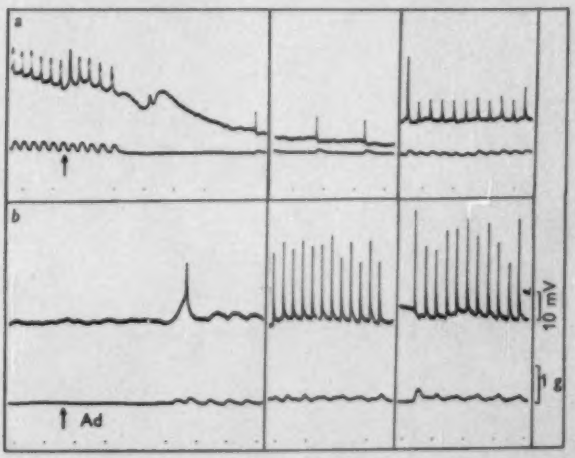

Fig. 9. The effect of adrenaline 5×10^{-7} on membrane potential, electrical activity and tension (*a*) in the presence of iodoacetate 10^{-6} for 38 min, (*b*) for 90 min. The second record in each row was taken 2 min, the third 5 min, after giving adrenaline (see text). Time marker 10 sec.

On the other hand, iodoacetic acid (IAA) 10^{-5} reduced not only the hyperpolarizing action of adrenaline but also its action on spontaneous spike activity and on the response to electrical stimulation. When IAA had abolished all spontaneous activity the effect of adrenaline was reversed. It now caused depolarization and initiated spikes (see Fig. 9).

Replacement of sodium

The experiments so far were consistent with the view that the activation of phosphorylase by adrenaline might increase the rate of energy supply for processes stabilizing the membrane, e.g. some of the energy might be utilized for the active extrusion of sodium. We therefore studied in three experiments the action of adrenaline in the absence of sodium from the medium. NaCl was replaced by LiCl and $NaHCO_3$ by $KHCO_3$ and a corresponding amount of KCl was withdrawn. The effects produced by substituting

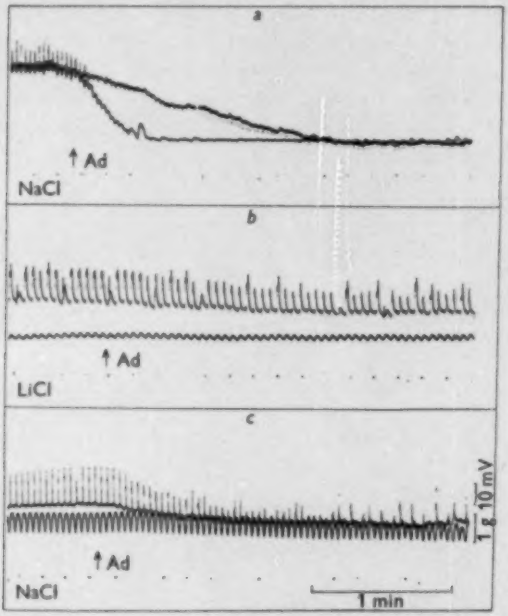

Fig. 10. The effect of adrenaline 5×10^{-7} on a preparation driven electrically (*a*) in normal solution; (*b*) 17 min after replacing NaCl by LiCl; (*c*) 85 min after replacing LiCl by NaCl.

Na with Li have been described elsewhere (Axelsson & Bülbring, 1959; Axelsson, 1960). Its main effect is to cause a decline of the tension response though it does not impair spike activity for several hours. Figure 10 shows records from an experiment in which the preparation was electrically driven. In normal solution (*a*) adrenaline abolished the conducted response to electrical stimulation, the tension fell to zero and the membrane was hyperpolarized. Substitution of NaCl by LiCl abolished the action of adrenaline almost completely (*b*), and even after 85 min in normal solution the adrenaline effect showed only a partial recovery (*c*).

DISCUSSION

The taenia coli of the guinea-pig, which was the intestinal smooth-muscle preparation used for the experiments, is a tissue possessing a high degree of spontaneous activity. Its membrane potential is very unstable and in normal conditions it probably never reaches a true resting value. Adrenaline relaxes this muscle because it stops the spontaneous spike discharge. It also causes an increase of the membrane potential, which may rise by as much as 20 mV.

We found that adrenaline increased phosphorylase activity in the smooth muscle of the taenia coli. When the biochemical effect was measured in parallel strips from the same taenia as that in which the biophysical changes were recorded, the increase in phosphorylase activity was seen at the same time as that at which the hyperpolarization was observed.

It may be assumed that the activation of phosphorylase in smooth muscle by adrenaline is brought about by a mechanism similar to that discovered by Sutherland & Rall (1958) in liver, i.e. by an increase in the formation of cyclic adenosine-3′,5′-phosphoric acid (3′,5′-AMP). This compound exerts a stimulating effect on the enzymic synthesis of active phosphorylase in liver (Rall, Sutherland & Berthet, 1957), as well as in skeletal and heart muscle (Rall & Sutherland, 1958; Krebs, Graves & Fisher, 1959). If the production of glucose-1-phosphate in taenia coli were limiting the rate of carbohydrate metabolism, activation of phosphorylase would increase the supply of metabolic energy.

Previous observations, especially those reported in the preceding paper (Axelsson & Bülbring, 1961), indicate that the metabolic rate may be an important factor influencing membrane excitability and controlling membrane polarization in smooth muscle. Our present results suggest that the increased supply of energy in the presence of adrenaline is utilized by processes in the cell membrane requiring energy.

Evidence for an accelerated rate of active Na extrusion in the presence of adrenaline has recently been obtained (E. Bülbring & P. J. Goodford, unpublished observations), when it was found that the rate of loss of ^{24}Na from taenia coli was approximately doubled by adrenaline, while the rate of uptake was reduced. Furthermore, we observed that adrenaline failed to produce its usual stabilizing effect when the Na in the medium was replaced by Li, which, as was shown by Keynes & Swan (1959) for frog skeletal muscle, is extruded much more slowly than Na.

An increased supply of energy by adrenaline activating phosphorylase should be prevented if the tissue was depleted of glycogen. It proved to be impossible to deplete the glycogen store completely. Even after 4 hr exposure to glucose-free solution there remained 20–25 % of the original

glycogen concentration in the muscle. This low level was reached in a shorter time, but it was not reduced further, in anaerobic conditions. It may be that the residual material is not identical with glycogen, but that it is another polysaccharide which cannot serve as a source of metabolic energy. If this were so, the failure of adrenaline to stabilize the membrane would be explained. In all experiments the action of adrenaline was profoundly altered, and in some reversed, when the preparation was exposed for many hours to glucose-free solution.

We may assume that, as long as there is glycogen available which can be utilized, adrenaline will accelerate its break-down. However, the resulting energy will be distributed between the contractile mechanism and the cell membrane. During the first stages of glucose depletion (see Fig. 4) adrenaline always produced hyperpolarization after a variable latency and to a variable degree. Its capacity to prevent spike discharge was diminished, but the tension could still be restored. When the glycogen store was presumably very low, and as is apparent from the figures shown in Table 3, the resulting portion was less easily broken down, adrenaline had a depolarizing action accelerating spike discharge (see Fig. 5). The available energy was first employed for the restoration of tension and only after a long latency it appeared to be available for the stabilization of the membrane. After still further depletion this metabolic action completely disappeared.

The striking effect of adrenaline during the period of recovery, when the preparation depended on adrenaline for a normal rhythmic activity, raises the question whether it might also exert an action on glucose uptake and utilization.

Of the metabolic inhibitors tested, iodoacetate appeared to be the most effective in abolishing the stabilizing effect of adrenaline on the cell membrane. Dinitrophenol (Burnstock, 1958) and azide only partially antagonized adrenaline.

A reversal of the action of adrenaline was observed in several conditions. After prolonged exposure to glucose-free solution and also in the presence of IAA adrenaline caused depolarization and initiated spikes. This result could be interpreted as a stimulant action—presumably by some change in membrane permeability—which was unmasked when the metabolic action had been abolished.

A few observations were recorded when adrenaline caused depolarization, but nevertheless stopped all spike activity. They occurred twice at an early stage in normal solution, twice at an early stage of exposure to glucose-free solution, and finally twice when, after exposure to low temperature, the preparation was warmed to 37° C. We have evidence that in these various conditions phosphorylase activity may be relatively high. In

those experiments in which we measured the effect of adrenaline on phosphorylase activity in parallel with observing its depolarizing action we found that adrenaline produced either no change or diminished phosphorylase activity. These results cannot as yet be explained and further experiments are required.

The remarkable coincidence between the hyperpolarization and the activation of phosphorylase in normal conditions supports the assumption of a causal relation. On the other hand, in some tissues 3′,5′-AMP and adrenaline increase the rates of other reactions concerned with the utilization of carbohydrate and of lipids (Cahill, Leboeuf & Flinn, 1960; Mansour & Menard, 1960; Rizak, 1960; Vaughan, 1960); therefore, biochemical mechanisms other than activation of phosphorylase may be associated with the hyperpolarizing action of adrenaline on taenia coli. Finally, the possibility cannot be excluded that 3′,5′-AMP may induce hyperpolarization by a direct effect on the membrane.

SUMMARY

1. Isolated guinea-pig taenia coli was used for recording biophysical changes from one strip and measuring phosphorylase activity in other strips subjected to the same experimental conditions.

2. In normal solution adrenaline stopped spontaneous spike activity, abolished the conducted response to electrical stimulation and caused hyperpolarization. At the same time adrenaline increased phosphorylase activity.

3. In glucose-free solution, as the preparation was gradually depleted of glycogen the stabilizing effect of adrenaline on the cell membrane and the hyperpolarization became gradually less, until, after prolonged exposure to glucose-free medium, the effect of adrenaline was reversed. It caused depolarization and initiated spikes.

4. A similar stimulant action was observed in the presence of iodoacetic acid which abolished the inhibitory action of adrenaline.

5. Cooling to 5° C abolished the hyperpolarizing action of adrenaline. When the preparation was warmed again to 37° C and phosphorylase activity was relatively high, adrenaline diminished it and caused depolarization.

6. The stabilizing effect on the cell membrane normally produced by adrenaline was not seen when sodium in the solution was substituted by lithium.

7. The results are consistent with the view that adrenaline has a dual action. It appears to have a direct depolarizing effect on the membrane which, however, is normally masked by its more powerful inhibitory action.

The experiments suggest that adrenaline exerts its inhibitory effect by increasing the rate of energy for active stabilizing processes at the cell membrane.

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CHLORIDE IONS AND THE MEMBRANE POTENTIAL OF PURKINJE FIBRES

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According to the ionic theory of electrical activity the membrane potential depends on the unequal distribution of ions between the intra- and extracellular phases, and on the relative permeability of the membrane towards them. For cardiac muscle the role played by sodium and potassium has been studied to some extent (see Hoffman & Cranefield, 1960) but that of chloride has received little attention. The present paper deals with the effects produced by the substitution for chloride ions of nitrate ions or large anions such as acetylglycinate and pyroglutamate. The results suggest that the contribution of chloride ions to the total membrane conductance is low in a resting fibre but increases when a fibre is depolarized. The findings and interpretations are in good agreement with those recently published by Hutter & Noble (1959). The main results of the present investigation have been reported in a preliminary communication (Carmeliet, 1959).

METHODS

Sheep hearts were obtained at the slaughterhouse and carried to the laboratory in cool Tyrode solution. 'False tendons' were removed from the left ventricle and kept in Tyrode solution at 37° C.

Membrane potentials were measured by two Ling–Gerard micro-electrodes (1949), one inside, the other outside the fibre. The electrodes were filled with 3 M-KCl and had a resistance between 10 and 20 MΩ. They were connected to the cathode-follower input of a differential amplifier by chlorided silver wires.

In a series of experiments the relative membrane resistance was measured by passing current through a second micro-electrode within the same fibre. Square current pulses of about 100 msec duration were used which hyperpolarized the membrane, at a frequency of 4/sec. The distance separating the intracellular electrodes was small (50–100 μ) compared to the space constant of a Purkinje fibre (2 mm; Weidmann, 1952). The amplitude of the recorded potential changes could thus be squared to obtain relative values for the membrane resistance (Hodgkin & Huxley, 1947).

In order to make measurements of membrane resistance at different membrane potentials the fibre was depolarized or hyperpolarized by means of a pair of external electrodes, as shown in Fig. 1. The bundle was pushed into a close-fitting hole in a Perspex block and solution was allowed to siphon along this part of the preparation. The two micro-electrodes were inserted at a distance of 1 mm from the hole. To insure that the recording micro-

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electrode was measuring the true membrane potential change, the reference electrode was so placed that with the recording micro-electrode just outside the fibre there was no potential change when current was switched on.

The composition of the normal Tyrode was as follows (mM): Na 149·9; K 2·7; Ca 1·8; Mg 0·5; Cl 145; HCO_3 11·9; H_2PO_4 0·32; glucose 1 g/l. The solution, when aerated by 95% O_2 and 5% CO_2, had a pH of 7·4. To study the influence of anions, NaCl was replaced by Na-acetylglycinate (Light) or Na-pyroglutamate (Light), or $NaNO_3$. The K^+ concentration was reduced by omission of KCl, and increased up to 50 mM by replacement of an equivalent amount of the NaCl. In sodium-free solutions, NaCl was replaced by choline chloride (Hoffmann-La Roche) on a mole-for-mole basis, and atropine sulphate was added (10 mg/l.). Choline acetylglycinate was used as a substitute for NaCl, in an attempt to combine a slowly penetrating cation with a slowly penetrating anion. This was prepared by neutralizing acetylglycine with choline hydroxide (Schuchardt). Choline nitrate was prepared from choline chloride, by adding $AgNO_3$ in an amount slightly less than equivalent, and filtering.

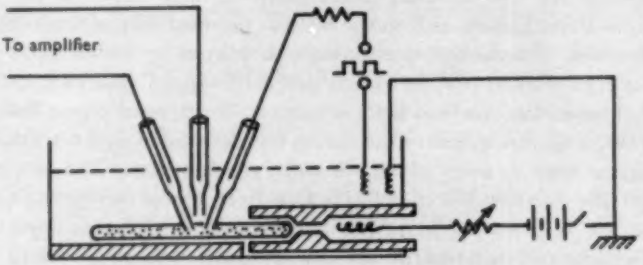

Fig. 1. Diagram of arrangement to measure the relative membrane resistance at different membrane potentials. Two intracellular micro-electrodes, one to record the membrane potential, the other to pass square wave current pulses of about 100 msec duration. The bundle was pushed into a close-fitting hole in a Perspex block, which served as an external polarizing electrode.

RESULTS

Effects of replacing chloride ions by other anions

The substitution of NaCl by Na-pyroglutamate or Na-acetylglycinate had the following effects (Figs. 2 and 3): (*a*) the frequency of spontaneously beating preparations went up temporarily, owing to a higher rate of the diastolic depolarization; (*b*) spontaneous activity often started with preparations that had previously been quiescent; (*c*) the plateau of the action potential was markedly prolonged; its slope was decreased, sometimes resulting in a standstill on the plateau level (−50 mV); (*d*) the plateau was also elevated, and this was most pronounced if the preparation was artificially stimulated at a high rate.

The substitution of chloride ions by nitrate ions produced the opposite effects: (*a*) the frequency of spontaneous activity was reduced, owing to a lower rate of the diastolic depolarization; (*b*) at the end of 5–10 min spontaneous activity ceased, and the fibre depolarized by about 10 mV,

to reach a stable membrane potential near −80 mV; (*c*) the plateau of the action potential was shortened, and (*d*) was brought closer to the resting potential (see Fig. 3). On returning from nitrate to chloride solution the action potentials initially lengthened beyond their previous duration to return to 'normal' with a half time of about 5 min.

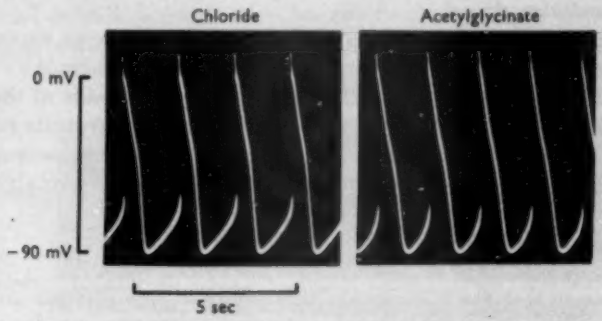

Fig. 2. Spontaneous action potentials in chloride-Tyrode (left) and in acetyl-glycinate-Tyrode (right). The rate of diastolic depolarization increased in the acetyl-glycinate solution.

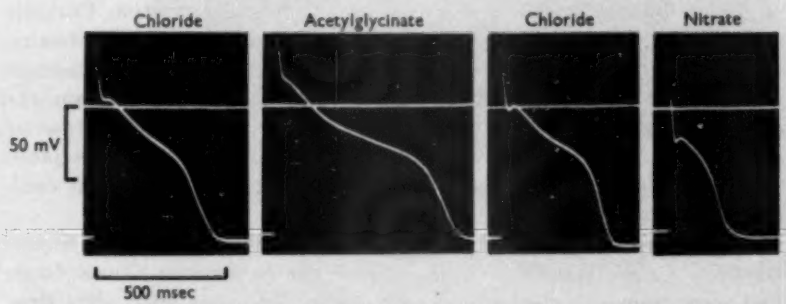

Fig. 3. Action potentials in chloride-Tyrode are compared with action potentials in acetylglycinate and nitrate solutions. The spike has been retouched.

In contrast to the considerable influence of anions on the shape of the action potential, substitution of chloride by large anions had no effect on the value of the maximum diastolic potential. To eliminate complications due to spontaneous activity chloride substitution was also studied in the absence of Na ions. Replacement of choline chloride by choline acetyl-glycinate did not change the value of the resting potential. This remained true also if the potassium concentration was increased from normal (2·7 mM) to 5·4, 13·5 or 50 mM.

For the sake of simplicity in the presentation of further results it may now be helpful to put forward a possible explanation. It is assumed (i) that

the membrane is permeable to anions, the permeability decreasing according to the series NO_3 > Cl > acetylglycinate; (ii) that the permeability to other ions is relatively large at the resting potential, but becomes comparable to that of chloride ions when the membrane is depolarized; and (iii) that chloride ions are passively distributed between the intra- and extracellular phases.

With this hypothesis in mind the observed effects may be explained in the following way. When the membrane is depolarized there is an inward current of chloride ions. This will tend to make the outside of the membrane more positive, and thus assists repolarization. With nitrate replacing chloride, this current will be increased, resulting in a shorter action potential and in a lower rate of diastolic depolarization. In the acetylglycinate solution the opposite effect will occur.

Chloride substitution in a potassium-free medium

In order to test the assumption that chloride ions play an important part in carrying electric charge when the membrane becomes depolarized, the effect of chloride substitution was studied in a potassium-free medium. While myocardial fibres hyperpolarize or do not change their membrane potential if potassium ions are omitted from the bathing solution, Purkinje fibres show a strong depolarization to a level near −50 mV (Weidmann, 1956). Flux measurements with ^{42}K indicate a decrease of potassium permeability (Carmeliet, 1960); Na ions are necessary to obtain the depolarization. So we have to deal with a situation in which potassium permeability is decreased, whilst the sodium permeability is appreciable. The potential in a K-free solution may be stable at −40 mV or may oscillate between −50 and 0 mV.

When chloride ions were replaced by acetylglycinate there was a further tendency for the membrane to depolarize: the oscillations became larger and more frequent; they would continue or disappear after some time. The resemblance to the influence of chloride substitution on the diastolic depolarization was obvious. On returning to the chloride-Tyrode solution without potassium, the oscillations disappeared and the membrane potential returned slowly towards −50 mV. Then suddenly, within a fraction of a second, the potential returned to −90 mV (Fig. 4). This full repolarization was followed by a train of spontaneous action potentials of normal configuration, but with increasing duration, until the membrane failed to repolarize and the potential became stable again at the −50 mV plateau level.

These results may be explained on the three assumptions stated above. Thus, when the fibre is depolarized in a K-free solution, the chloride ions redistribute according to the new potential. If chloride ions are now

replaced by acetylglycinate ions the outflow of chloride ions outweighs the inflow of the larger anions. The net outflow of negative charge explains the larger and more frequent oscillations. Readmitting the chloride ions results in an inward flow of negative charge, thus the tendency to repolarize. However, the potential returns to the plateau level (-50 mV) as soon as the internal chloride concentration has risen sufficiently to slow down the net entry of chloride ions. It is noteworthy that readmission of chloride

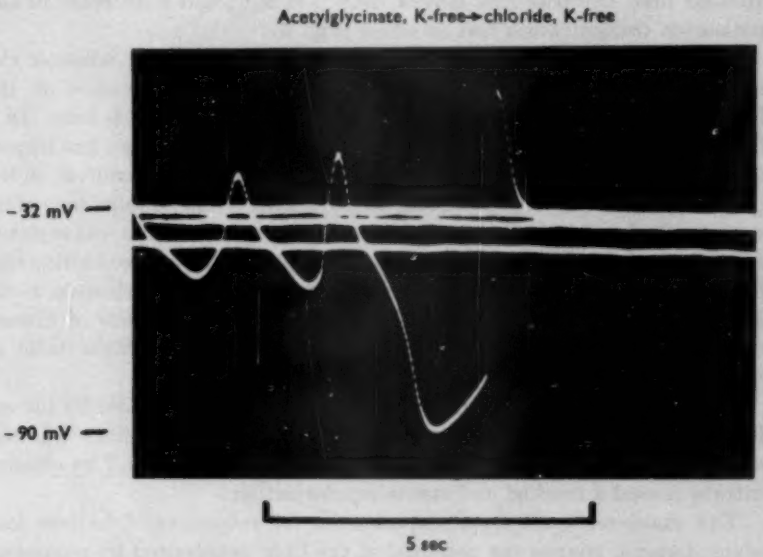

Fig. 4. Effect of replacing acetylglycinate by chloride ions in a potassium-free medium. The membrane was first depolarized in a potassium-free acetylglycinate medium to -32 mV. Readmitting chloride ions brought about a transitory repolarization to -90 mV.

ions has a larger effect than chloride deprivation. This may be explained by the difference in the relative change of the chloride concentration gradient and by regenerative phenomena: during the flow of hyperpolarizing current a decrease in sodium permeability could be coupled with an increase in potassium permeability, both favouring repolarization.

While nitrate ions hardly increased the resting potential when the normal potassium concentration was used, they caused a hyperpolarization of more than 20 mV if the membrane was first depolarized by potassium lack. Reverting to the normal chloride concentration resulted in a marked depolarization. This again can be explained by a larger permeability to nitrate than to chloride ions.

Chloride substitution in a potassium- and sodium-free solution

H. H. Hecht & S. Weidmann had found (unpublished observations) that Purkinje fibres in a sodium- and potassium-free solution could show two different stable potentials, −90 and −50 mV. When potassium was omitted first there was a depolarization to −50 mV, and the reduction of sodium ions did not change the potential further. When sodium was omitted first, the potential stayed near −90 mV, and a decrease in the potassium concentration had no effect (Fig. 5).

The following experiments were undertaken to find out whether the influence of the sequence of potassium and sodium deprivation on the final result depended on the stabilizing effect of the chloride ions. In a first series of experiments the 'chloride battery' was rendered less important by working in a Cl-free (acetylglycinate) medium. Removal of the potassium ions led to depolarization, as usual. When sodium ions were now omitted and, simultaneously, the chloride concentration was restored to normal, the potential returned to −90 mV (Fig. 6). If the chloride ions had been readmitted in the presence of sodium ions the repolarization would have been transitory (see preceding section). In the absence of inward current due to the sodium ions, however, the potential became stable at the high value.

In a second series of experiments chloride ions were replaced by nitrate ions. The sequence is illustrated in Fig. 6. A Purkinje fibre was first depolarized in a potassium-free solution. Replacing the NaCl by choline nitrate caused a marked and stable repolarization.

The main result of these experiments is: reduction of sodium ions alone does not change the potential of the fibre depolarized by potassium lack to any large extent. If, however, at the same time there exists a disequilibrium for the anion distribution, the membrane can be fully repolarized. The permeability to anions in the depolarized state seems to be relatively important.

Polarizing current in a potassium- and sodium-free solution

If the chloride ions really distribute themselves passively it ought to be possible to change the intracellular concentration by altering the membrane potential for some time. After the break of current applied from an external source, the membrane potential should have a tendency, thanks to the redistribution of the chloride ions, to stay near the value that has been imposed.

The membrane potential was changed by means of current flowing through an intracellular electrode; the potential change was recorded by a second intracellular electrode. In order to obtain a fairly uniform

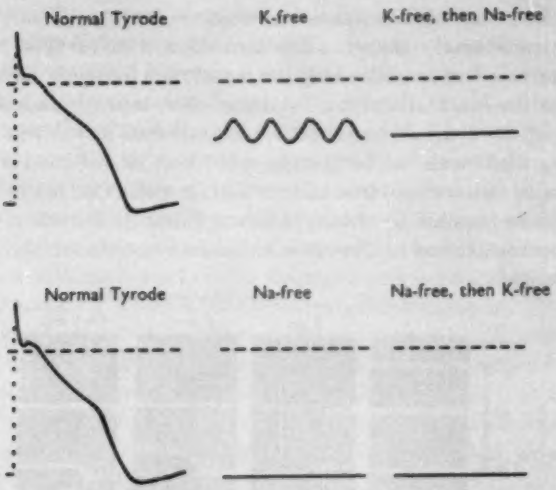

Fig. 5. Tracings of the transmembrane potential of a sheep Purkinje fibre: the sequence of potassium and sodium depletion determined the potential finally obtained.

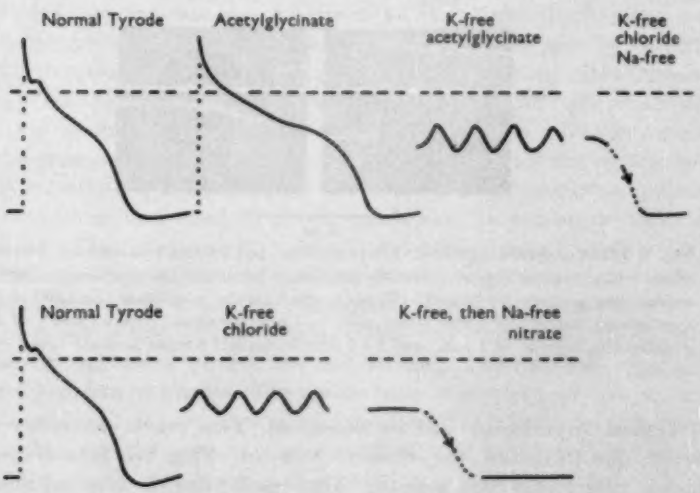

Fig. 6. Tracings of the membrane potentials of a sheep Purkinje fibre: influence of the disequilibrium for anions in a potassium- and sodium-free medium. For explanation see text.

change over the whole surface of the membrane it was necessary to use short pieces (order of one space constant, = 2 mm). If any biological cable is cut transversely, there is a depolarization, which is most pronounced at the cut surface but spreads along the membrane for some distance due to the fact that the extracellular fluid and the 'core' represent a low resistance to the flow of short-circuit currents. In an empirical way it was found that short pieces with 'seals' at both ends could best be obtained if the second cut was made in a sodium-free and potassium-rich (13·5 mM) medium. In practice it was possible to obtain pieces of Purkinje fibres 3 mm in length which, when transferred to Tyrode solution and stimulated, showed normal action potentials.

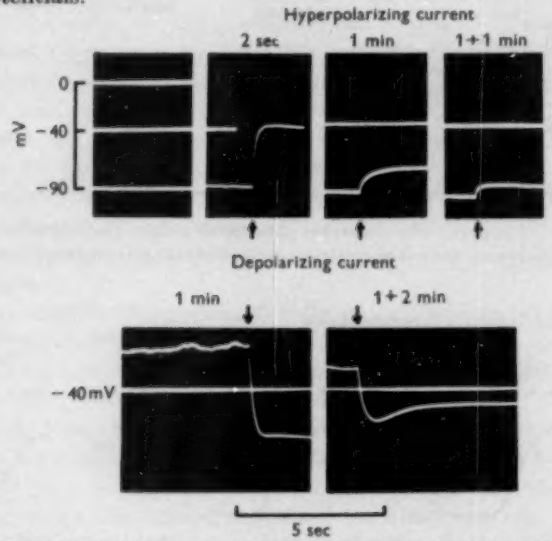

Fig. 7. Effect of polarizing current in a potassium- and sodium-free solution. Above (from left to right): the two possible membrane potentials in a potassium- and sodium-free medium (−40 and −90 mV); effect on the membrane potential of a repolarizing current of 2 sec, 1 min and 1+1 min duration. Below: effect of a depolarizing current of 1 min, and 1+2 min duration. Arrows indicate break of current.

A typical experiment will be described. Two micro-electrodes were inserted, one to record, the other to polarize. First the potassium was reduced, afterwards the sodium. The result was a depolarization to −40 mV. Figure 7 illustrates the effects of then applying hyperpolarizing currents for different periods. At the break of a hyperpolarizing current lasting for 2 sec the potential returned quickly to the −40 mV level. After passing a hyperpolarizing current for 1 min, however, the potential

did not return to the initial level but stayed at an intermediate value. A hyperpolarizing current reapplied for a further minute resulted in a full and stable repolarization to −90 mV. Soon afterwards current was passed in the opposite direction. A depolarizing current lasting for 1 min caused only a slight reduction in potential below −90 mV. Having passed current for another 2 min the potential stayed at the −50 mV level.

It is thus possible to move the potential from one stable level to the other, by imposing on the membrane that new potential value for a few minutes. The current must be carried by ions that are present within the intra- and extracellular phases. An inward current must be carried by positive ions moving inwards or by negative ions moving outwards. There was no sodium or potassium in the extracellular solution. The only ions moving inwards could be choline, magnesium or calcium, but it is hard to see how an inward flux of these ions could stabilize the potential at the −90 mV level. However, it seems reasonable to suggest that the current is carried by Cl ions moving outwards. With a decrease of the internal chloride concentration the chloride equilibrium potential would tend to approach the −90 mV level.

The effect of anions on the membrane resistance

A change in the electrical membrane resistance when an ion concentration is altered may indicate the importance of that particular ion as a carrier of membrane current. It had been reported in an abstract by Weidmann (1955) that the electrical membrane resistance of Purkinje fibres, measured 2 sec after the make of a polarizing current, was a function of the membrane potential. To simplify the system, sodium-free solutions were used in the present study. As can be seen in Figs. 8 and 9 the resistance was maximal between −20 and −40 mV. For reversed membrane potentials the resistance decreased to about quarter of its maximum value near +40 mV. The resistance at −90 mV depended strongly on the external potassium concentration and could be smaller (high potassium concentration) or larger (low potassium concentration) than the value at +40 mV.

The effect of the chloride concentration was studied at different potassium concentrations (0, 2·7, 5·4 and 50 mM). Chloride was replaced by acetylglycinate or nitrate. The results were dependent on the potassium concentration. At potassium concentrations of 0, 2·7 and 5·4 mM, replacement of chloride by acetylglycinate resulted in an increase of the membrane resistance if the membrane was depolarized beyond −50 mV. Figure 8 shows an example at 0 mM potassium. The increase was to about twice for potentials between 0 and +50 mV. At potentials between −50 and −100 mV it was hard to see any difference. In some experiments there was a slight increase of resistance in the acetylglycinate solution, but it

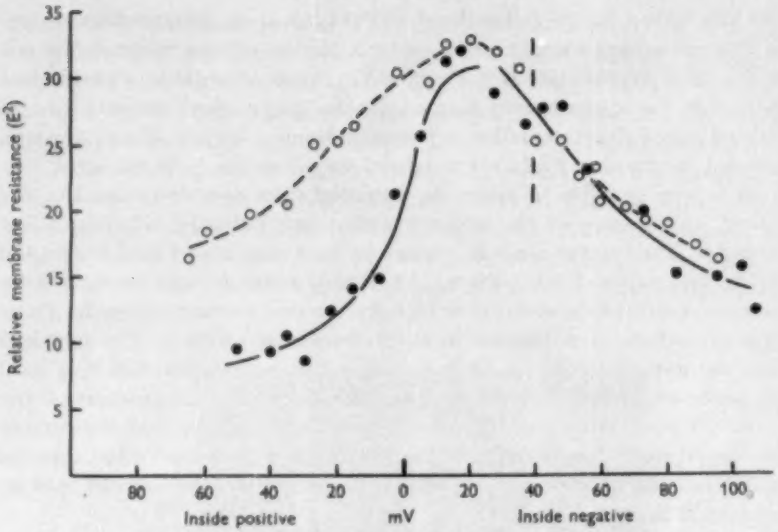

Fig. 8. Relative membrane resistance as a function of the membrane potential. ●, chloride medium; ○, acetylglycinate medium. The vertical interrupted line indicates the membrane potential without polarizing current; the extracellular potassium concentration was 0 mM. For large depolarizations (−20 to +60 mV inside potential), the membrane resistance in the acetylglycinate medium was higher than in the chloride medium.

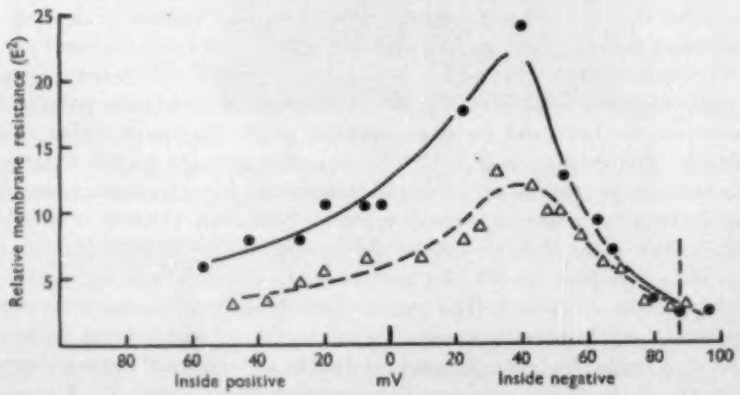

Fig. 9. Relative membrane resistance as a function of the membrane potential. ●, chloride medium; △, nitrate medium. The vertical interrupted line indicates the membrane potential without polarizing current; the extracellular potassium concentration was 5·4 mM. Between −40 and +60 mV the resistance was decreased in the nitrate-Tyrode. Note that the resistance at −90 mV was lower than at +40 mV, while the reverse was true in Fig. 8. This is due to the difference in potassium concentration.

was impossible to be sure that these differences were not due to regenerative phenomena or to small differences in the estimated membrane potential.

The reverse result was observed in the nitrate solution (Fig. 9, potassium concentration 5·4 mM). The membrane resistance was roughly halved between −20 and +50 mV, but was unchanged between −50 and −100 mV.

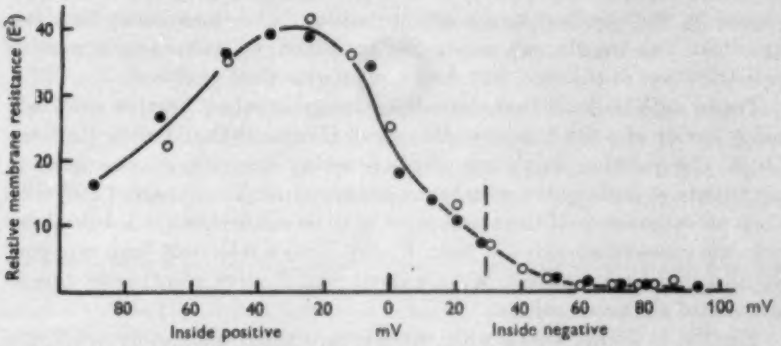

Fig. 10. Relative membrane resistance as a function of the membrane potential. ●, chloride medium; ○, acetylglycinate medium. Vertical interrupted line, membrane potential without polarizing current. Potassium concentration was 50 mM. There was no difference between the two curves.

At a potassium concentration of 50 mM there was no difference at any membrane potential between the chloride and the acetylglycinate solution (Fig. 10). It might be stressed that the anomalous rectification found in the normal Tyrode solution was also present in high potassium concentrations, and was even more pronounced.

The conditions under which chloride ions become important as carriers for current are thus: (*a*) depolarization, (*b*) a low external potassium concentration.

DISCUSSION

From the point of view of describing electrical events during the cardiac cycle, the conclusion of the present experiments is thus: in resting fibres, chloride ions play but a minor role; however, when the fibres are depolarized, as during activity, chloride ions become important as carriers of electrical charge. This seems to be due to a decrease in K conductance of the surface membrane upon depolarization rather than to an increase in Cl conductance.

The question as to whether or not chloride ions are distributed as 'passive' ions between the intra- and the extracellular phases cannot be

answered in a final way. The intracellular Cl concentration corresponding to a resting potential of −90 mV and an extracellular Cl concentration of 145 mM would be 5 mM. Analytical data for intracellular chloride are difficult to obtain since the methods depend on a correct estimation of the extracellular space. It is felt that the analytical figures so far published cannot be used as evidence either for or against the idea of a passive distribution (Robertson & Peyser, 1956; Lamb, 1960). It was reported in the present paper that the membrane potential can be permanently displaced (p. 380) by imposing a new potential on the membrane for a few minutes. This result can easily be explained by assuming a passive redistribution of chloride, but does not provide final evidence.

Tracer data indicate that chloride exchange in rabbit heart is relatively quick (order of a few minutes, Manery & Haege, 1941; Sekul & Holland, 1959). The question thus arises why in a resting fibre chloride was found to contribute so little to the membrane conductance. The apparent difficulty finds an explanation if the assumption may be made that the intracellular chloride concentration is low (near 5 mM). Thus a relatively high rate constant would be compatible with a small contribution of chloride ions as carriers of electrical charge.

Finally, it seems worth while to compare some membrane properties of skeletal and cardiac muscle. In both tissues inward movement of chloride ions during the action potential has been shown to contribute to repolarization (Etzensperger & Bretonneau, 1956; Falk & Landa, 1960; Hutter & Noble, 1960). On the other hand a number of differences exist: (*a*) for a resting membrane the chloride conductance is relatively small in cardiac tissue and large in skeletal muscle (Giebisch, Kraupp, Pillat & Stormann, 1957; Hodgkin & Horowicz, 1959; Hutter & Padsha, 1959); (*b*) this difference is still present when the extracellular potassium concentration is increased (Hodgkin & Horowicz, 1959; Adrian, 1960); (*c*) nitrate ions penetrate more slowly than chloride ions in skeletal muscle (Hutter & Padsha, 1959), while this order is reversed in cardiac muscle.

SUMMARY

1. Na acetylglycinate, Na pyroglutamate and $NaNO_3$ were used as substitutes for NaCl in order to study the influence of anions on the membrane potential and membrane resistance of Purkinje fibres of sheep hearts.

2. The anions had no influence on the maximum diastolic potential, in normal Tyrode solution (2·7 mM-K) and on the resting potential at higher potassium concentrations (5·4, 13·5, and 50 mM).

3. The rate of diastolic depolarization was increased in acetylglycinate and decreased in nitrate. The action potential was lengthened and the

plateau elevated by large anions; in nitrate the action potential was shortened and the plateau was closer to the resting potential.

4. In potassium-free or potassium- and sodium-free solutions the membrane potential could be changed markedly by an anion disequilibrium. In the potassium- and sodium-free solution it was possible to displace the membrane potential permanently by imposing a new potential difference on the membrane for a few minutes.

5. At membrane potentials between -20 and $+50$ mV the electrical membrane resistance was increased in the acetylglycinate and decreased in the nitrate solution. At membrane potentials between -50 and -100 mV there was no change.

6. The results support the hypothesis that (*a*) chloride ions are passively distributed, (*b*) chloride ions contribute but little to the total membrane conductance at the resting potential in normal Tyrode solution (-90 mV), but (*c*) become important as electrical charge carriers when the membrane is depolarized.

I wish to express my indebtedness to Professor S. Weidmann for his valuable help and criticism during this work. My thanks are also due to Professor A. von Muralt for the hospitality at the Physiological Institute of Berne. My stay in Berne was made possible by a traveller fellowship of the Belgian government and a grant (no. 1138) to Professor S. Weidmann from the Swiss National Science Foundation.

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EFFECT OF BARIUM IONS ON MEMBRANE POTENTIALS OF COCKROACH GIANT AXONS

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There is evidence that barium ions may be substituted for sodium ions to produce action potentials in crustacean muscle fibres (Fatt & Ginsborg, 1958) and in mammalian B and C fibres (Greengard & Straub, 1959). In these fibres the action potentials are remarkably prolonged in an isotonic barium solution. In mammalian A fibres, however, barium cannot be substituted for sodium, and isotonic barium causes conduction block (Greengard & Straub, 1959).

A series of investigations on the excitation and electrical properties of cockroach giant axons has revealed that they resemble in many respects the giant axons of the squid, which have been widely regarded as representative of non-myelinated nerve fibres (Narahashi, 1960*a*). For example, the membrane potential of cockroach giant axons behaves quite similarly to that of squid giant axons with various external concentrations of sodium and potassium (Yamasaki & Narahashi, 1958, 1959*a*), and the negative after-potential can be explained on the same basis (Narahashi & Yamasaki, 1960*a*). It is therefore interesting to examine the behaviour of the membrane potential of this non-myelinated nerve fibre under the influence of barium.

Attention has recently been focused on the nature of the after-potential as well as on that of the spike potential. There have appeared many publications dealing with the augmentation and prolongation of the negative after-potential under a variety of experimental conditions (see Shanes, 1958). In the giant axons of the cockroach the negative after-potential is increased by treatment with calcium-rich solutions and the insecticides dicophanum (DDT) and allethrin (synthetic pyrethrin) (Narahashi, 1960*a*, *b*; Narahashi & Yamasaki, 1960*b*, *c*). Barium also increases the negative after-potential of the giant axon of the cockroach and an attempt was made to elucidate the mechanism of this increase.

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METHODS

The giant axons in the abdominal nerve cord of the American cockroach, *Periplaneta americana* L., were used throughout the experiments. The methods of stimulation and recording were essentially similar to those described in the previous paper (Narahashi & Yamasaki, 1960*a*). External stimulation was effected by a pair of silver-wire electrodes, while recordings were made by a micro-electrode inserted in the desheathed region of an axon. In the present experiments, however, an additional micro-electrode was often inserted in the same axon within 50 μ of the recording electrode in order to deliver polarizing current. The voltage drop across a resistor connected in series with the current-passing electrode gave a measure of current strength. Micro-electrodes having a resistance of 10–20 MΩ were selected for both polarization and recording. The input stray capacity was compensated by a negative-capacity feedback amplifier, the rising phase of a recorded square pulse of current being reduced to less than 10 μsec when the above-mentioned micro-electrodes were used.

TABLE 1. Composition of solutions (mM)

	Na	Ba	Sucrose	K	Ca	Cl	HCO_3
Normal	214	0	0	3·1	1·8	220·7	0·5
150 mM-Na	150	0	128	3·1	1·8	156·7	0·5
150 mM-Na + 21 mM-Ba	150	21	64	3·1	1·8	198·7	0·5
150 mM-Na + 43 mM-Ba	150	43	0	3·1	1·8	242·7	0·5
100 mM-Na	100	0	227	3·1	1·8	106·7	0·5
100 mM-Na + 21 mM-Ba	100	21	163	3·1	1·8	148·7	0·5
100 mM-Na + 43 mM-Ba	100	43	98	3·1	1·8	192·7	0·5
100 mM-Na + 76 mM-Ba	100	76	0	3·1	1·8	258·7	0·5
75 mM-Na	75	0	278	3·1	1·8	81·7	0·5
75 mM-Na + 93 mM-Ba	75	93	0	3·1	1·8	267·7	0·5
50 mM-Na	50	0	328	3·1	1·8	56·7	0·5
50 mM-Na + 109 mM-Ba	50	109	0	3·1	1·8	274·7	0·5
Isotonic Ba	0	143	0	3·1	1·8	292·7	0·5

pH: ca. 7·2.

The Ringer's solution was the same as that described in the previous paper (Narahashi & Yamasaki, 1960*a*) except that phosphates were replaced by bicarbonate as a buffer. When the effect of barium was to be examined, sodium was replaced by barium, keeping the solution isotonic. A sucrose Ringer's solution in which the corresponding amount of sodium was replaced by sucrose was used as a control in addition to normal Ringer's solution. The concentrations of potassium and calcium were kept constant in all modified Ringer's solutions, including isotonic Na-free Ba-Ringer's solution. The composition of the solutions used is given in Table 1.

Since the micro-electrodes were kept inserted while the bathing fluid was changed by continuous perfusion, as described previously (Narahashi & Yamasaki, 1960*a*), the resting potential in the test solutions was measured in the following way. The input circuit, when an axon is impaled by the recording micro-electrode consists of the following: grid of cathode follower—Ag-AgCl—3 M-KCl (micro-electrode)—axoplasm—nerve membrane—bathing fluid—agar-Ringer—3 M-KCl—Ag-AgCl—ground. While the recording micro-electrode was kept inserted in an axon, the input of the recording system was grounded at intervals and the resulting difference in potential, 'apparent resting potential', was measured. This value was the sum of the resting potential, the tip potential of micro-electrode, and the potentials produced at the other interphases of the input circuit. The latter potentials included those produced at the junctions between the Ag-AgCl and the

3 M-KCl in the micro-electrode, between the bathing fluid and the agar-Ringer of the indifferent electrode, between the agar-Ringer and the 3 M-KCl in the indifferent electrode, and between the 3 M-KCl and the Ag-AgCl of the indifferent electrode. At the end of each experiment the recording micro-electrode was withdrawn in normal Ringer's solution and the potential change was measured. This value gave the resting potential measured directly at that moment. Furthermore, the input was grounded while the tip of the micro-electrode just withdrawn from the axon was in normal Ringer's solution, and the potential change was measured. This value gave the sum of the tip potential and the other junction potentials, and was therefore subtracted from all measurements of the apparent resting potential made during the course of the experiment. Since the resistance of the micro-electrode was relatively low, it could be assumed that the magnitude of the tip potential measured in axoplasm was the same as it was in bathing fluid, and that the tip potential was constant throughout the experiment. The measurements in test solutions needed an additional correction. The junction potential between the bathing solution and the agar-Ringer of the indifferent electrode should have changed when the ionic composition of bathing solution was altered. The difference in junction potential was measured in another set of experiments with the test solutions given in Table 1. The recording micro-electrode was replaced by a glass tube of about 8 mm inside diameter in which agar-3-M-KCl and 3 M-KCl solutions were introduced. The input was grounded while the KCl tube was dipped in the bathing fluid, and the potential change was measured. Such a potential obtained in normal Ringer's solution was then subtracted from the potentials in the test solutions. Although the differences were only a few millivolts, they were further subtracted from the apparent resting potentials to obtain the true resting potentials. Despite these corrections, the measurements of the resting potential were less reliable than those of the action potential.

The experiments were conducted during the summer months at room temperatures ranging from 28 to 34·5° C. Although the temperatures were somewhat high, they did not have any harmful effect either on the intact cockroaches or on the isolated nerve cord preparations, the resting and action potentials remaining nearly constant in normal Ringer's solution for over 5 hr.

RESULTS

Resting and action potentials

Isotonic Ba solution. When normal Ringer's solution was replaced with isotonic barium solution, nerve conduction was immediately blocked, but the effect was completely reversible upon washing with normal Ringer's solution.

Solution with 75 mM-Na and 93 mM-Ba. Nerve conduction was also blocked when the concentration of barium was reduced to 93 mM, as is illustrated in Fig. 1. The normal Ringer's solution containing 214 mM-Na was first replaced by 75 mM-Na containing no Ba (records *A* and *B*). The magnitude of the action potential was markedly reduced, and the rate of rise and the rate of fall of the action potential, especially the former, were also decreased, but the positive phase which followed the spike phase remained almost unchanged. This observation is in good agreement with that made previously (Yamasaki & Narahashi, 1958, 1959*a*). When the bathing fluid was then changed to 75 mM-Na + 93 mM-Ba, the action potential became double-peaked and finally conduction was blocked, leaving only a small residual potential (record *C*). The graded nature of

the change was undoubtedly due to slow exchange of the bathing fluid. The double-peaked action potential is likely to be a manifestation of saltatory conduction across the desheathed region of the nerve cord where the micro-electrode is inserted, because the nerve sheath provides a strong

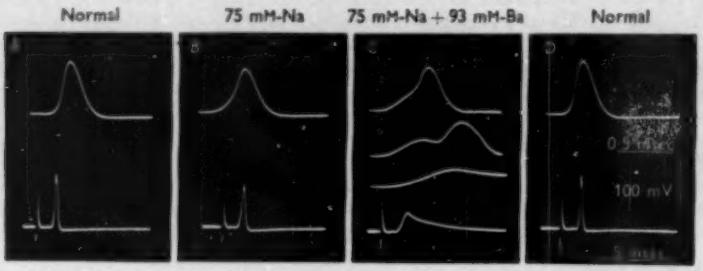

Fig. 1. Records of the action potential showing the effect of reducing Na and adding Ba. In column *C* three records were taken with fast sweep during the course of and after conduction block, and the bottom record was taken with slow sweep after conduction block. Axon 10*d*. Temperature 31·5° C.

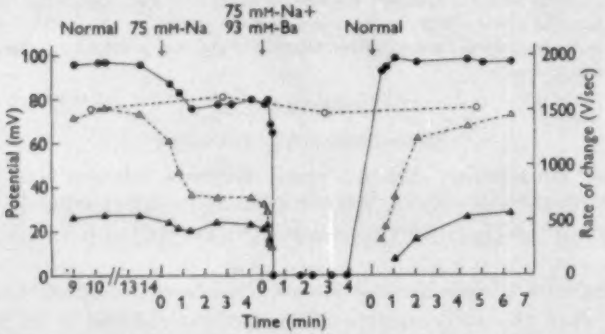

Fig. 2. Changes in the resting potential (○), and in the amplitude (●), the maximum rates of rise (△) and of fall (▲) of the action potential by a reduction of Na and an addition of Ba. Axon 10*d*. Temperature 31·5° C.

barrier against the penetration of ions (Yamasaki & Narahashi, 1959*a*). The effect was completely reversible on washing with normal Ringer's solution (record *D*). An example of the full course of an experiment is presented in Fig. 2, in which the magnitudes of the resting and action potentials and the maximum rates of rise and fall of the action potential are plotted against time. The resting potential was slightly increased by

75 mM-Na and slightly decreased by 75 mM-Na + 93 mM-Ba, but the decrease was too small to account for the conduction block.

Solutions with 100 mM-Na and various concentrations of Ba. The effects of various concentrations of barium contained in 100 mM-Na Ringer's solution are shown in Figs. 3 and 4. Replacement of normal Ringer's solution with 100 mM-Na caused the spike amplitude to decrease. When 21 mM-Ba was added to 100 mM-Na, the resting potential was somewhat

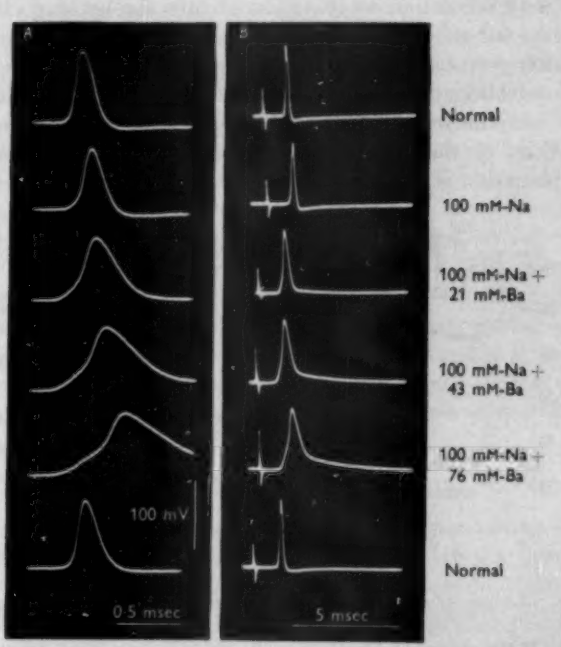

Fig. 3. Records of the action potential in various concentrations of Ba. Both fast (*A*) and slow (*B*) sweep records are shown in each solution. Axon 13*b*. Temperature 34·5° C.

reduced, whereas the height of the action potential increased slightly. Both rising and falling phases of the action potential were slowed down considerably. Another conspicuous change was the reversal of the slow phase following the spike potential, the positive phase being converted into a negative after-potential. Increase in barium concentration to 43 mM and then to 76 mM brought about further accentuation of these effects, except for the effect on the spike amplitude which was nearly unchanged. At these concentrations of barium there appeared a remarkable step on the rising phase of the action potential (Fig. 3). This can be accounted for by

supposing that an action potential originating in the intact region of the nerve cord, where barium has not yet come into contact with the axon because of the barrier of the nerve sheath, spreads electrotonically along the axon to the point of impalement where the nerve has been desheathed and thereby is under the influence of barium, and that the critical depolarization for firing is greatly augmented by barium, as will be described in a later section. All these effects of barium were completely reversed when normal Ringer's solution was introduced into the bathing chamber. The mean values for the amplitude and the maximum rates of rise and fall of the action potential are given in Table 2.

It is interesting to note that the amplitude of the action potential increased temporarily above normal upon readmission of normal Ringer's solution (Fig. 4) Such a tendency was observed very often. The most likely explanation is as follows. When the perfusion of normal Ringer's

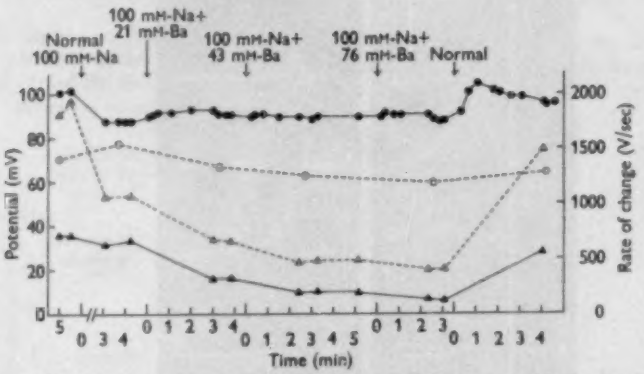

Fig. 4. Changes in the resting potential (○), and in the amplitude (●), the maximum rates of rise (△) and fall (▲) of the action potential in various concentrations of Ba. Axon 13*b*. Temperature 34·5° C.

TABLE 2. Mean values for the amplitude, and the maximum rates of rise and fall of the action potential in various concentrations of Na and Ba. The numbers of measurements are shown in parentheses

	Na concentration (mM)	Ba concentration (mM)			
		0	21	43	76
Amplitude (mV)	214	104 (31)	—	—	—
	150	94 (18)	110 (1)	97 (21)	—
	100	91 (11)	93 (4)	92 (4)	90 (11)
Maximum rate of rise (V/sec)	214	1790 (72)	—	—	—
	150	1350 (8)	800 (1)	790 (7)	—
	100	980 (11)	610 (4)	470 (4)	370 (10)
Maximum rate of fall (V/sec)	214	660 (27)	—	—	—
	150	590 (8)	430 (1)	320 (7)	—
	100	610 (11)	360 (4)	250 (4)	210 (10)

solution is begun, the sodium concentration is progressively raised while the barium concentration is progressively lowered. If the rate at which the action potential was affected by the change in sodium concentration were higher than that at which it was affected by the change in barium concentration, then the rising phase, which is known to be more affected by [Na] than the falling phase (Yamasaki & Narahashi, 1959*a*), would approach the normal level earlier, whereas the falling phase would remain slow both in velocity and in its onset. This would result in an extension of the rising phase beyond its normal level, thereby causing the spike amplitude to increase. After a while, however, the effect of barium on the falling phase would disappear, so that the action potential would revert to its normal shape and magnitude.

Repetitive excitation was rarely induced by a single shock in the barium-treated axons.

Solutions with 150 mM-Na and various concentrations of Ba. Experiments similar to those described in the preceding section were carried out using 21 mM- and 43 mM-Ba each contained in 150 mM-Ba. The effect of the barium was essentially the same as before the rising and falling phases of the action potential being slowed down, and the negative after-potential being considerably increased. The increase in spike amplitude by barium was also observed and was more marked than it was in 100 mM-Na. The mean values for the amplitude and the maximum rates of rise and fall of the action potential are given in Table 2.

Critical depolarization and threshold current

Pulses of depolarizing currents of 3 msec duration were delivered through a micro-electrode, and the resulting potentials and the current strengths were recorded. An example of records taken in 150 mM-Na is illustrated in Fig. 5*A*. When the current strength approached threshold, graded responses appeared which terminated in a slight undershoot. At threshold an action potential arose from the top of the graded response and terminated in a marked undershoot. The inflexion point between the slowly rising catelectrotonic potential and the action potential was measured from the resting level and was taken as a measure of the critical depolarization for firing. There was a general tendency for the spike amplitude to become smaller and for the critical depolarization to become slightly higher as the current intensity was reduced. These phenomena are explicable in terms of the inactivation caused by longer lasting cathodal current flow (Hodgkin & Huxley, 1952*a*).

In 150 mM-Na + 43 mM-Ba the critical depolarization was greater, and no undershoot followed the spike, as is shown in Fig. 5*B*.

The mean values for the threshold current and the critical depolariza-

tion are given in Table 3. The critical depolarization increased from 25 to 31 mV on reducing the sodium concentration from 214 mM (normal Ringer) to 150 mM, and concomitantly the threshold current increased from $3{\cdot}4 \times 10^{-8}$ A to $6{\cdot}3 \times 10^{-8}$ A. Barium caused a further increase in critical depolarization to 40 mV, but it had hardly any effect on the threshold current.

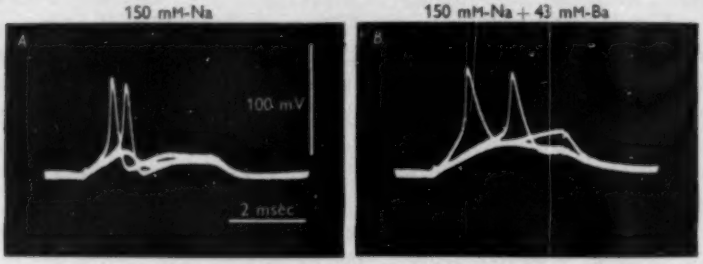

Fig. 5. Records of the catelectrotonic potential and action potential in 150 mM-Na and in 150 mM-Na + 43 mM-Ba. Axon 26. Temperature 32·5° C.

TABLE 3. Critical depolarization and threshold current. Each value represents the mean obtained for one axon by several repeated measurements, the number of which is shown in parentheses. In some axons, several sets of measurements were made at intervals, and the mean values for each set are presented separately

		Normal Ringer's solution		150 mM-Na		150 mM-Na + 43 mM-Ba	
Axon no.	Temp. (°C)	Critical depol. (mV)	Threshold current ($\times 10^{-8}$ A)	Critical depol. (mV)	Threshold current ($\times 10^{-8}$ A)	Critical depol. (mV)	Threshold current ($\times 10^{-8}$ A)
20*a*	29·5	24 (13)	—	—	—	—	—
20*b*	29·5	—	—	—	—	39 (10)	—
		—	—	—	—	36 (5)	—
21	28	24 (5)	3·4 (5)	—	—	—	—
22	28	24 (7)	3·4 (3)	—	—	43 (7)	4·4 (7)
		28 (5)	—	—	—	—	—
23	31	—	—	32 (8)	8·6 (8)	43 (10)	9·3 (5)
24	31	—	—	32 (6)	6·3 (4)	—	—
25	32·5	—	—	33 (7)	6·8 (5)	—	—
26	32·5	—	—	26 (5)	5·0 (5)	42 (4)	4·6 (4)
		—	—	32 (8)	5·0 (8)	41 (4)	5·2 (4)
28	30	—	—	28 (4)	—	35 (4)	—
Mean	30·2	25	3·4	31	6·3	40	5·9

Action potential as affected by the displacement of membrane potential

The total current passing through the nerve membrane can be divided into a capacity current and an ionic current. Thus we have

$$I = C\frac{dV}{dt} + I_i,$$

where I is the total membrane current density, I_i is the ionic current density, C is the membrane capacity per unit area, V is the displacement of the membrane potential from its resting value and t is time (Hodgkin & Huxley, 1952*b*). In the case where an action potential is propagated along an axon of uniform structure bathed in a large volume of conducting fluid, the membrane current density is expressed as

$$I = \frac{a}{2R_i\theta^2}\frac{d^2V}{dt^2},$$

where a is the radius of the axon, R_i is the specific resistance of the axoplasm and θ is the velocity of conduction (Hodgkin & Huxley, 1952*b*). Hence,

$$I_i = \frac{a}{2R_i\theta^2}\frac{d^2V}{dt^2} - C\frac{dV}{dt}.$$

Therefore, when the rate of rise of the action potential is at a maximum,

$$I_i = -C\frac{dV}{dt}.$$

This means that the ionic current density is proportional to the maximum rate of change of the action potential. In the giant axon of the squid, the inward current upon depolarization is known to be related to the membrane potential immediately before depolarization (Hodgkin & Huxley, 1952*a*). It follows that the rate of rise of the action potential, which is indicative of the inward sodium current, should also be related to the membrane potential from which the action potential arises. This is actually the case in Purkinje fibres (Weidman, 1955).

Now it has been shown that barium causes a slight but definite depolarization. Simultaneously, the rising and falling phases of the spike potential were slowed down. It is therefore reasonable to suppose that the slow rising phase in barium might be due to the fall of resting potential and if this were the case, the rate of rise would be restored by hyperpolarizing the membrane.

Figure 6 illustrates a series of records of the action potential in depolarized (records *A* and *C*) and in hyperpolarized (records *B* and *D*) membranes. In Fig. 7, the height, the maximum rate of rise and the maximum rate of fall of the action potential obtained in normal Ringer's solution and in 150 mM-Na + 43 mM-Ba solution are plotted against the displacement of the membrane potential.

In normal Ringer's solution, the peak of the action potential fell short of the normal level as depolarization became greater (Fig. 6*A*, *b–d*), whereas it could reach almost the normal level even when the membrane was hyperpolarized to a considerable extent (Fig. 6*B*, *a–c*). However,

when the membrane was hyperpolarized still further, the peak of the action potential fell short of the normal level (*Bd*). These effects are more clearly seen in Fig. 7, in which the spike heights measured in normal Ringer's solution (filled-in circles) fall close to a straight line having a slope of about unity in the hyperpolarized membrane, whereas the line bends slightly upwards in the depolarized membrane. The rate of rise of the action potential was lowered very effectively by depolarization (Fig. 6*A* and Fig. 7, filled triangles) this being in good agreement with previous

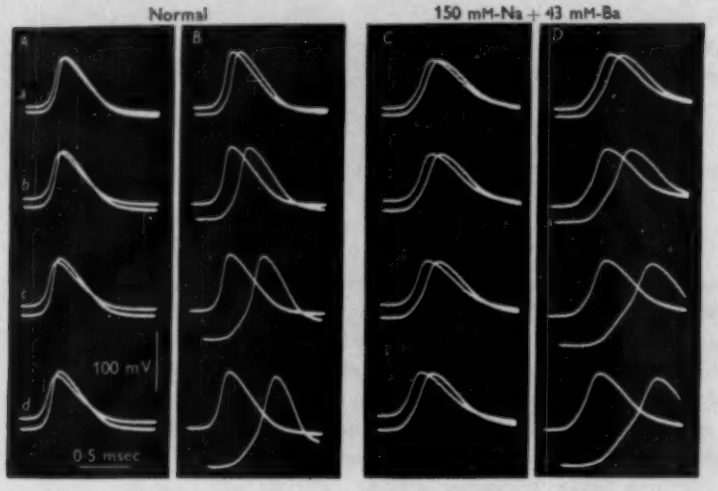

Fig. 6. Effects of depolarization (*A* and *C*) and hyperpolarization (*B* and *D*) on the action potential in normal Ringer's solution and in 150 mM-Na + 43 mM-Ba. The action potential was produced by external stimulation about 20 msec after the onset of polarizing current. In *A* and *C*, the upper trace in each record represents the response in depolarized membrane, and in *B* and *D*, the lower trace is the response in hyperpolarized membrane. Axon 19. Temperature 29·5° C.

observations (Yamasaki & Narahashi, 1959*b*). The rate of rise was also somewhat slowed down by hyperpolarization, although such a slowing was not observed previously (Yamasaki & Narahashi, 1959*b*). The explanation for the discrepancy is not immediately obvious though higher temperature in the present experiments is likely to be one of the factors involved.

On treatment with 150 mM-Na + 43 mM-Ba, there appeared two changes worthy of note. In the first place, the depressive effect of depolarization on the spike height disappeared. The peak of the action potential could now reach its normal level even when the maximum depolarizing current was applied (Fig. 6*C*), so that the curve relating the spike height to the

displacement of the membrane potential became a straight line both in depolarized and in hyperpolarized membranes (Fig. 7, open circles). In the second place, the rate of rise of the action potential became more stable than normal in the sense that the relation with the membrane potential became a straight line with a steep slope, the rising phase now being slightly accelerated by depolarization (Fig. 7, open triangles).

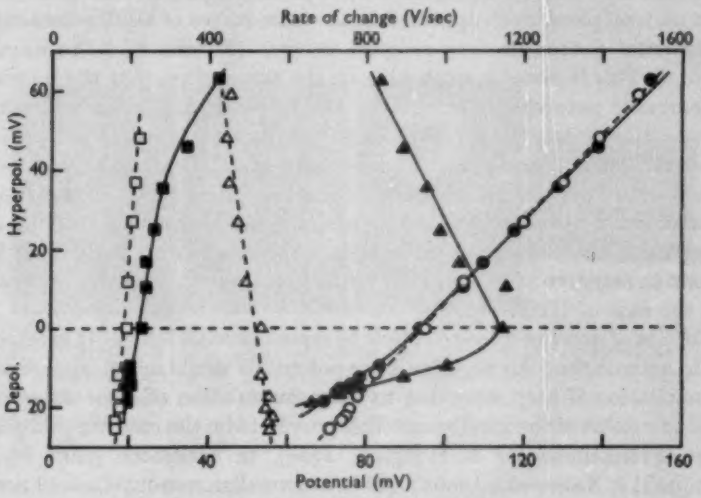

Fig. 7. Changes in the amplitude (○●), and in the maximum rates of rise (△▲) and fall (□■) of the action potential caused by the displacement of the membrane potential from the resting level. The filled symbols represent the measurements in normal Ringer's solution, and the open ones in 150 mM-Na + 43 mM-Ba. Axon 19. Temperature 29·5° C.

Although the slowness of the rise of the action potential seems to indicate a reduction of the sodium-carrying system rapidly available upon depolarization, these two features are both well explained by supposing that the inactivation of the sodium-carrying system normally occurring upon depolarization does not take place so effectively as in the normal axons. These observations rule out the possibility that the slowing of the rising phase of the action potential caused by barium is due to the fall of resting potential. There was no marked difference in the rate of fall of the action potential between the normal and Ba-treated axons, except that the absolute value was smaller in the latter (Fig. 7, open squares) than in the former (filled-in squares).

Nature of the negative after-potential increased by Ba

There is good evidence that the negative after-potential of the cockroach giant axon bathed in normal Ringer's solution can be explained by the accumulation of released potassium in the immediate vicinity of the nerve membrane (Narahashi & Yamasaki, 1960*a*). The insecticide DDT has been shown to cause the negative after-potential to increase markedly, and a plateau phase even appeared in the later stages of DDT poisoning if the external potassium was reduced to zero (Narahashi & Yamasaki, 1960*b*, *c*). This increase is explicable on the assumption that the increase in membrane potassium conductance taking place during the latter half of the action potential is delayed and partly suppressed (Narahashi & Yamasaki, 1960*b*), and that in later stages of DDT poisoning a partial suppression of sodium inactivation during the falling phase of the action potential may also be involved (Narahashi & Yamasaki, 1960*c*). An attempt was therefore made to elucidate the mechanism underlying the increase in negative after-potential by barium through the same approach as in the case of DDT.

Addition of negative after-potentials by repetitive stimulation. It is reasonable to assume that the negative after-potentials would add during repetitive excitation if they were due to the accumulation of some substance inside or outside of the membrane. This proved to be the case in squid giant axons (Frankenhaeuser & Hodgkin, 1956), in cockroach giant axons (Narahashi & Yamasaki, 1960*a*) and in mammalian non-myelinated nerve fibres (Greengard & Straub, 1958), where the negative after-potential was explained by the accumulation of potassium near the membrane. On the other hand, the negative after-potentials would not add, on repetitive stimulation, if they were not caused by such an accumulation of any substance. Examples may be provided by DDT-poisoned cockroach giant axons, in which the negative after-potential is greatly augmented (Narahashi & Yamasaki, 1960*b*), and by skeletal muscle fibres of the frog (Ishiko & Sato, 1956)

The records of Fig. 8 were taken in 150 mM-Na + 43 mM-Ba. A large negative after-potential appeared after a single response (record *A*). Upon repetitive stimulation at a frequency of 200/sec, there was little, if any, addition of the negative after-potentials (record *B*). By increasing the stimulus frequency up to 500 /sec, there appeared a slight addition (record *C*). Such a small addition is rather comparable to that observed in the normal axon (Fig. 2 in Narahashi & Yamasaki, 1960*a*), and, therefore, can be accounted for by the accumulation of released potassium in the immediate vicinity of the nerve membrane.

The absence of marked addition of the negative after-potentials on

repetitive stimulation is more clearly seen in Fig. 9, in which the decay of the negative after-potentials of the first through fourth and sixteenth impulses are plotted on a semi-logarithmic scale against time as was done in the previous papers (Narahashi & Yamasaki, 1960*a*, *b*). The open circles show the calculated values for the negative after-potentials which would be expected if they added in a linear manner. The actual small addition of the negative after-potentials seen in this figure falls far short of the calculated addition.

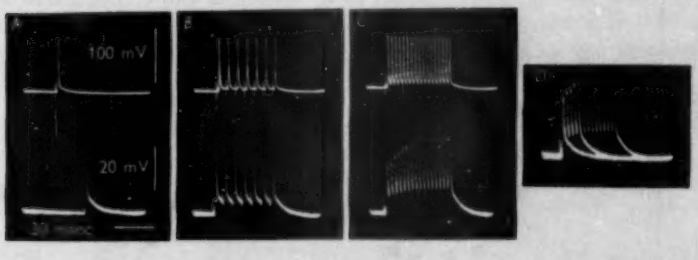

Fig. 8. Records of the action potentials produced by single and repetitive shocks in 150 mM-Na + 43 mM-Ba. The frequencies of repetitive stimulation are 200/sec in *B* and 500/sec in both *C* and *D*. Record *D* is a superimposed photograph with varying numbers of stimuli. The lower voltage calibration also applies to *D*. Axon 22. Temperature 28° C.

Time constant of membrane. The measurement of the time constant of decay of the negative after-potential should throw some light on the mechanism of its production, because it is of importance to know whether or not the decay is passive in nature. Record *D* in Fig. 8 is a superimposed photograph with varying numbers of stimuli. It seems at first sight that the decay is almost the same regardless of the number of impulses. Figure 9 reveals this point clearly. Unlike the normal axon, in which the negative after-potential could be expressed as a single exponential term (Narahashi & Yamasaki, 1960*a*), the initial exponential phase was terminated in a faster phase. The time constants of the initial phase in this particular fibre were 5·3 msec (1st impulse), 5·0 msec (2nd), 4·9 msec (3rd and 4th) and 4·6 msec (16th), and those of the terminal phase 3·1 msec (1st impulse), 3·0 msec (2nd) and 2·2 msec (3rd, 4th and 16th), there being a slight but definite tendency for both time constants to decrease with increasing number of stimuli. The same tendency was always seen in other Ba-treated fibres, and is in sharp contrast with the normal axon where the reverse tendency is observed (Narahashi & Yamasaki, 1960*a*). This problem will be discussed later.

The time constant of the initial falling phase of the negative after-potential in a single impulse is given in Table 4. The mean value is calculated as 4·7 msec.

In order to determine the resting membrane time constant of the Ba-treated axon, measurements were made on the rising phase of the

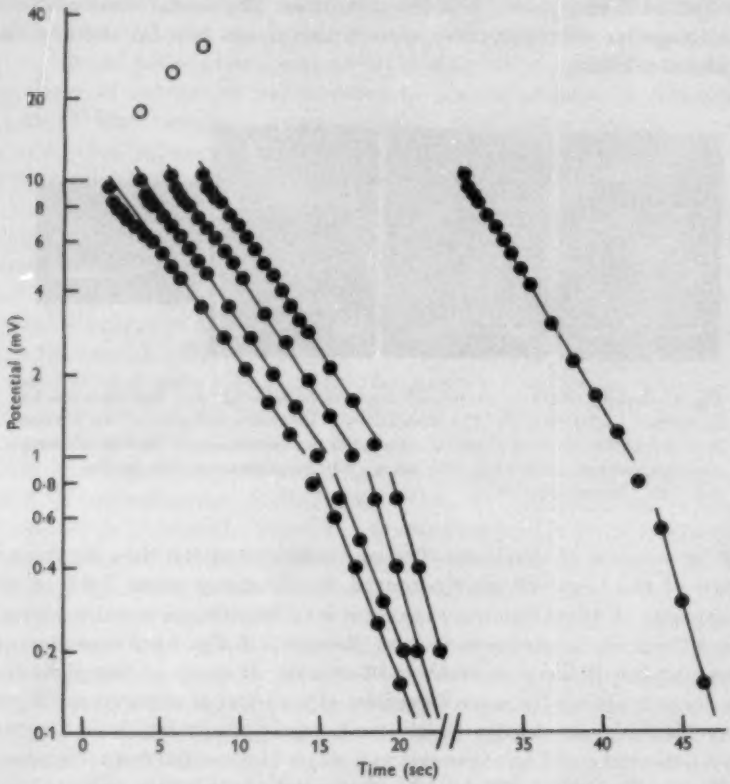

Fig. 9. Time course of the decay of the negative after-potential during a train of impulses at 500/sec in 150 mM-Na + 43 mM-Ba. The first, second, third, fourth and sixteenth impulses are illustrated. The ordinate represents the potential on a logarithmic scale, and the abscissa is the time measured from the beginning of the train. The filled circles are the measured points. The straight lines were drawn by eye. The time constants of the initial phase and of the terminal phase (in parentheses) are: 1st, 5·3 msec (3·1 msec); 2nd, 5·0 msec (3·0 msec); 3rd and 4th, 4·9 msec (2·2 msec); 16th, 4·6 msec (2·2 msec). The open circles represent the potential immediately after the end of the second, third and fourth spikes, calculated by adding the initial height of the first negative after-potential to the potential remaining at the moment when the next spike arises. Axon 22. Temperature 28° C.

anelectrotonic potential produced by a weak inward current. Anelectrotonic potentials having a steady-state magnitude of about 10 mV were chosen for measurements, because the membrane resistance became somewhat greater with increasing anodal current, thereby making the time constant larger than its resting value. The time to reach 84 % of the steady-state magnitude was taken as a measure of the time constant, because the time course of the electrotonic potential was regarded as an error function (Hodgkin & Rushton, 1946) under the present experimental conditions. The time constants are shown in Table 4, the mean value being 14·0 msec. This value is nearly three times as large as the time constant of the negative after-potential, which suggests that the falling phase of the negative after-potential is affected by some accelerating mechanism.

Table 4. Time constants (msec)

Axon no.	Temp. (°C)	Decay of negative after-potential	Decay of catelectrotonic potential of various amplitudes				Rise of anelectrotonic potential
			2 mV	5 mV	10 mV	20 mV	
18	29·5	3·7	6·5	5·9	4·8	2·6	14·4
19	29·5	4·4	4·3	4·0	3·4	2·3	15·5
22	28	4·5	6·0	5·6	5·1	4·1	10·7
		4·1	7·0	7·0	7·0	7·0	—
		5·3	8·1	7·8	7·3	6·4	15·7
26	32·5	6·0	5·9	5·7	5·4	4·4	16·2
28	30	5·2	3·4	3·4	3·4	3·4	11·6
		—	3·5	3·4	3·3	3·1	—
Mean	29·9	4·7	5·6	5·4	5·0	4·2	14·0

The time constant of decay of the negative after-potential in a single impulse was obtained from the slope of the initial phase of decay drawn on a semi-logarithmic scale as shown in Fig. 9. The time to fall to 16 % of the steady-state amplitude was taken as a measure of the constant of decay of the catelectrotonic potential. The measurements of the catelectrotonic potentials with various magnitudes were plotted against depolarization as in Fig. 11, and the values at each depolarization listed were obtained from the straight line of the graph. The time to reach 84 % of the steady-state amplitude was taken as a measure of the time constant of rise of the anelectrotonic potential. About 10 mV hyperpolarization was employed.

As the next step, the time constant was measured on the falling phase of a catelectrotonic potential. A series of records of catelectrotonic potentials with varying magnitudes was taken as shown in Fig. 10. The time constants measured from such records are plotted against depolarization in Fig. 11. It is seen that they become smaller with depolarization in a linear manner. From such a straight line, the time constants were picked out at depolarizations of 2, 5, 10 and 20 mV, and are listed in Table 4. There is agreement in the order of size of the time constant between the negative after-potential and the catelectrotonic potential, both of which had much smaller time constants than that of the anelectrotonic potential. This seems to imply

that the acceleration of the falling phase of the negative after-potential is related to a depolarization of the membrane.

Voltage–current relation. From the data so far described it seems probable that the mechanism by which the falling phase of the action potential is accelerated is somewhat inhibited by barium, thereby causing the

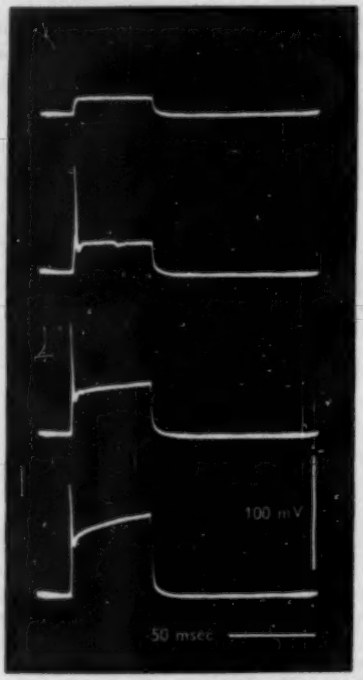

Fig. 10. Records of catelectrotonic potentials of various magnitudes in 150 mM-Na + 43 mM-Ba. Axon 28. Temperature 30° C.

negative after-potential to increase. The mechanism presumably involves the rise in membrane potassium conductance and the inactivation of the sodium-carrying system (Hodgkin & Huxley, 1952*b*). If any effect on the rise in potassium conductance were responsible for the increase in negative after-potential, the voltage–current relation would be changed.

Figure 12 shows a series of records of the catelectrotonic and anelectrotonic potentials in 150 mM-Na (records *A* and *B* respectively) and in 150 mM–Na + 43 mM-Ba (records *C* and *D*). The membrane showed clearly a delayed rectification in 150 mM-Na, which is in accordance with the previous observations (Yamasaki & Narahashi, 1959*b*). It should be

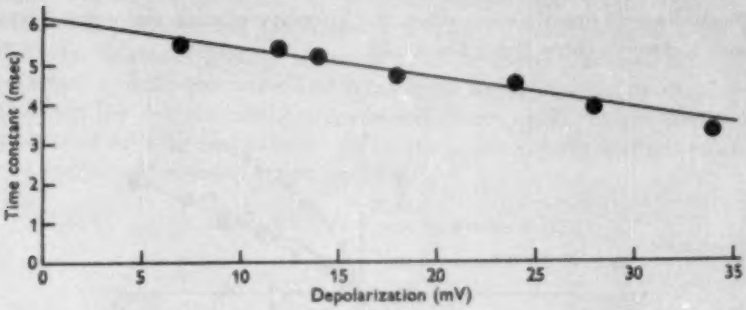

Fig. 11. The relation between the magnitude of depolarization (abscissa) and the time constant of decay of the catelectrotonic potential (ordinate) in 150 mM-Na + 43 mM-Ba. The straight line was drawn by eye. Axon 26. Temperature 32·5° C.

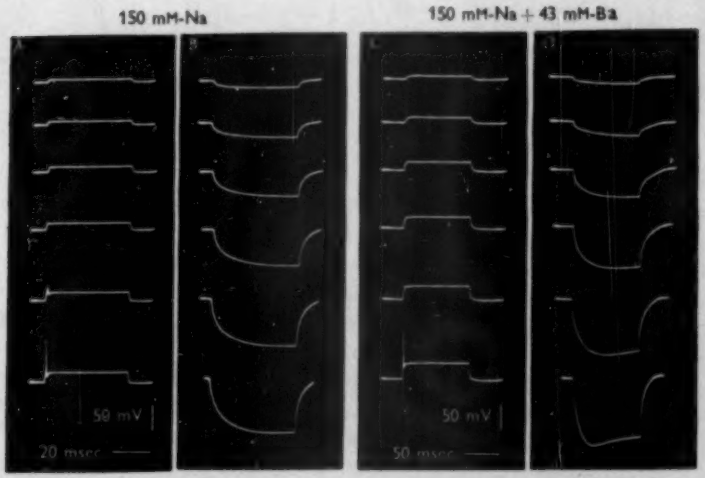

Fig. 12. Records of the catelectrotonic (*A* and *C*) and anelectrotonic (*B* and *D*) potentials in 150 mM-Na and in 150 mM-Na + 43 mM-Ba. The current strengths were: *A* (from top to bottom), 1·15, 2·18, 2·87, 3·91, 5·17, and $6{\cdot}90 \times 10^{-6}$ A; *B*, 1·38, 2·07, 3·27, 4·60, 5·75, and $7{\cdot}98 \times 10^{-6}$ A; *C*, 0·58, 0·92, 1·97, 3·24, 4·64 and $6{\cdot}49 \times 10^{-6}$ A; *D*, 0·46, 0·92, 2·14, 3·48, 4·87 and $8{\cdot}44 \times 10^{-6}$ A. Axon 26. Temperature 32·5° C.

added that no repetitive excitation occurred either in 150 mM-Na or in 150 mM-Na + 43 mM-Ba even when the intensity of cathodal current was raised to two or three times threshold.

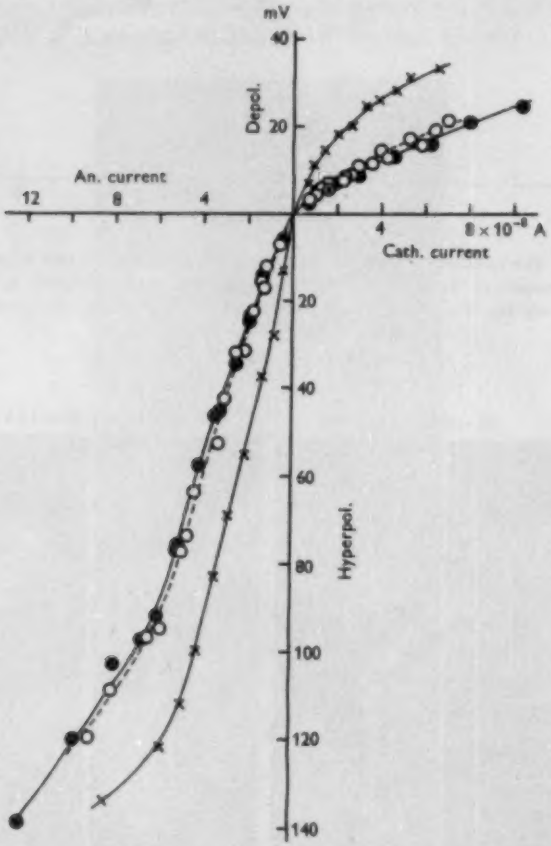

Fig. 13. Voltage–current relations. Measurements were made at first in 150 mM-Na (●), next in 150 mM-Na + 43 mM-Ba (×), and then again in 150 mM-Na (○). The abscissa represents the current strength and the ordinate the potential. Axon 26. Temperature 32·5° C.

The magnitudes of the steady-state electrotonic potentials are plotted against the current strengths in Fig. 13. The filled circles show the control measurements made in 150 mM-Na. It should be noted that the effective resistance across the membrane shown by the slope of the curve is reduced not only by depolarization but also by hyperpolarization greater

than about 100 mV. The crosses represent the measurements in 150 mM-Na + 43 mM-Ba. As is clearly shown by the change in slope, the effective resistance greatly increases both for depolarized and for hyperpolarized membranes, except at the largest hyperpolarization employed, although the delayed rectification is still observed. When the nerve was again washed with the 150 mM-Na solution, the voltage–current relation was completely restored (open circles).

TABLE 5. Effective membrane resistance (kΩ)

		At resting potential			At a depolarization of 10 mV			At a depolarization of 20 mV	
Axon no.	Temp. (°C)	Normal Ringer's solution	150 mM-Na	150 mM-Na + 43 mM-Ba	Normal Ringer's solution	150 mM-Na	150 mM-Na + 43 mM-Ba	150 mM-Na	150 mM-Na + 43 mM-Ba
21	28	620	—	—	220	—	—	—	—
22	28	770	—	2000	410	—	850	—	710
		1330	—	2500	520	—	820	—	600
23	31	—	660	1250	—	190	260	130	110
24	31	—	830	—	—	250	—	160	—
25	32·5	—	800	—	—	160	—	—	—
26	32·5	—	1100	—	—	250	—	210	—
		—	1270	2630	—	280	930	200	480
		—	1280	—	—	290	—	290	—
27*a*	30	—	800	—	—	170	—	110	—
27*b*	30	—	780	—	—	150	—	—	—
28	30	—	820	1950	—	260	970	160	290
		—	—	1610	—	—	490	—	240
Mean	30·3	910	930	1990	380	220	720	180	410

From voltage-current curves such as that shown in Fig. 13, effective resistances across the membrane were calculated and are listed in Table 5. The resting effective resistance refers to the slope at the point where no current is passed. The effective resistance was also measured at depolarizations of 10 and 20 mV. The increase in effective resistance caused by barium amounts to about twice in the resting membrane and about twice to three times in the depolarized membrane.

Since the membrane effective resistance (R_e) is related to membrane resistance (the resistance times unit length of the membrane, r_m) and axoplasm resistance (the resistance per unit length of the axoplasm, r_i) by the equation $R_e = \frac{1}{2}\sqrt{(r_m r_i)}$, the relative change in the membrane resistance caused by depolarization or hyperpolarization can be computed from the voltage–current curve, if the axoplasm resistance is assumed to remain constant. Such calculated curves are shown in Fig. 14, in which it is seen that the Ba-treated axon has a membrane resistance much higher than normal at all depolarizations.

An attempt was made to calculate current density from the voltage–current curve of Fig. 13. By the current density is meant current per unit area of membrane which would result from a uniform change in

potential (V) over the whole length of the fibre. According to Cole & Curtis (1941), the current density (i) is proportional to $I.\mathrm{d}I/\mathrm{d}V$, where I is current intensity passed through the membrane. As has been shown by Burke & Ginsborg (1956), if the specific resistance of the axoplasm (R_i) and the diameter of the fibre (d) are known,

$$i = \frac{R_i}{\pi^2 d^3} I \frac{\mathrm{d}I}{\mathrm{d}V}.$$

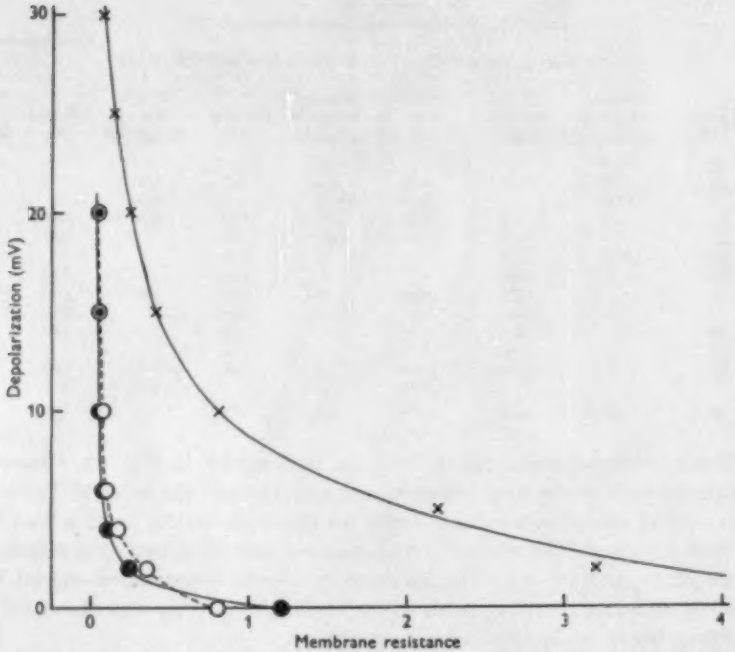

Fig. 14. The relation between membrane resistance in arbitrary units (abscissa) and the magnitude of depolarization (ordinate) calculated from the curves of Fig. 13. Measurements were made first in 150 mM-Na (●), next in 150 mM-Na + 43 mM-Ba (×), and then again in 150 mM-Na (○).

The calculated curves are drawn in Fig. 15, in which the abscissa represents the displacement of the membrane potential from the resting level, and the ordinate the current density in arbitrary units. If the specific axoplasm resistance and the fibre diameter are assumed to be 130 Ω cm and 50 μ respectively (Yamasaki & Narahashi, 1959*b*), each unit of current density would be equivalent to about 10 μA/cm^2. The current density is shown to be very much reduced by barium both in depolarized and in hyperpolarized membranes.

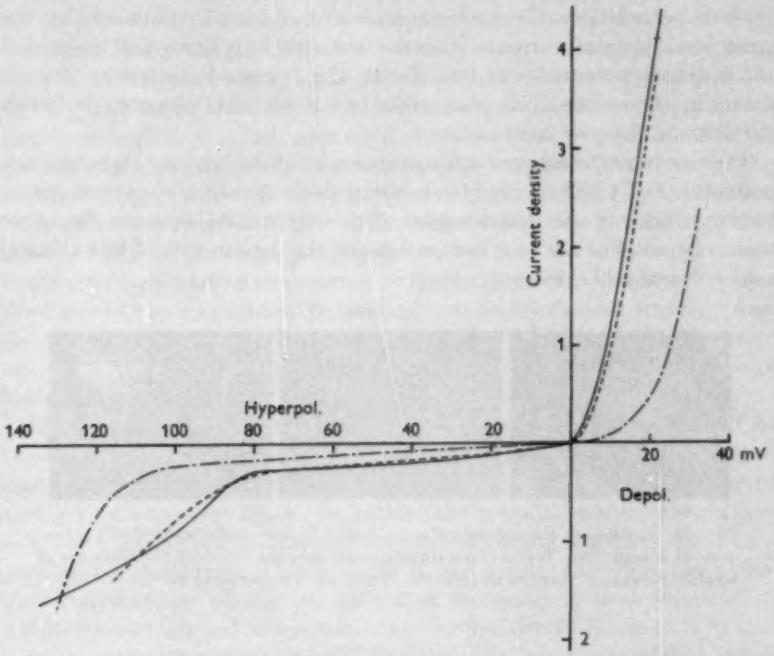

Fig. 15. The relation between the displacement of membrane potential from the resting level (abscissa) and the current density (ordinate) in arbitrary units, i.e. currents per unit area of membrane which would result from a uniform change in potential over the whole length of the axon. The curves were calculated from Fig. 13. Measurements were made first in 150 mM-Na (———), next in 150 mM-Na + 43 mM-Ba (—·—·—), and then again in 150 mM-Na (– – – –).

Transmembrane stimulation in isotonic Ba solutions

It has already been pointed out that the isotonic barium medium causes conduction block. However, it remains to be seen whether under these conditions the membrane is capable of producing responses that fail to conduct along the axon. This problem has not been examined in detail, and only the results of preliminary experiments will be described here.

Records of the electrotonic potential of the axon bathed in isotonic barium are shown in Fig. 16. No spike potential was generated even when the membrane was depolarized by as much as 120 mV (record *A*). Record *B* in Fig. 16, which was taken with a faster sweep, shows the rising phase of the catelectrotonic potential more clearly. There is a step in the early part of the rising phase which at first sight might seem to be a local

response. However, this possibility was excluded by superposing the catelectrotonic potential on the anelectrotonic one (record *C*) produced by the same but opposite currents, because both rising phases just coincided and no extra potential was seen during the course of the catelectrotonic potential. Therefore, it is reasonable to assume that the step indicates the onset of delayed rectification.

The small potentials seen during the course of the largest catelectrotonic potential (Fig. 16 *B*) are likely to be electrotonic spread of the action potentials produced in the intact region of the nerve cord, because the nerve sheath behaves as a strong barrier against the penetration of ions (Yamasaki & Narahashi, 1959*a*).

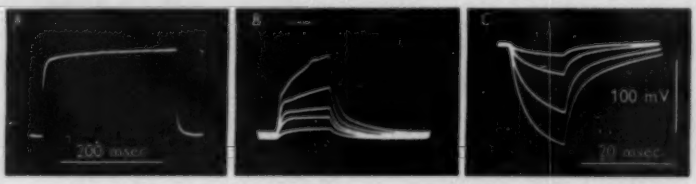

Fig. 16. Records of the catelectrotonic (*A* and *B*) and anelectrotonic (*C*) potentials in isotonic Ba. *B* and *C* are superimposed records, in which the intensity of applied current is changed at random. Axon 28. Temperature 30° C.

DISCUSSION

Unlike crustacean muscle fibres (Fatt & Ginsborg, 1958) and mammalian B and C fibres (Greengard & Straub, 1959), the giant axons of the cockroach are unable to produce action potentials in an isotonic barium solution resembling in this respect mammalian A fibres (Greengard & Straub, 1959). However, the action of barium, to increase and prolong the falling phase of the action potential seen with crustacean muscles and mammalian B and C fibres, has also been demonstrated in cockroach giant axons with rather weak concentrations of barium. The increase in the negative afterpotential caused by weak concentrations of barium was also observed in frog nerve (Lorente de Nó & Feng, 1946).

The increase in resting membrane resistance caused by barium is consistent with the fall of resting potential. Assuming that the resting membrane conductance is largely but not exclusively due to potassium conductance (Yamasaki & Narahashi, 1959*a*), the decrease in the latter should cause depolarization.

It seems likely that the slowness of the rise of the action potential caused by barium is due to a depression of the sodium-carrying system. The rise in the critical level of depolarization is consistent with this view.

The fact that the active membrane potential and the rate of rise of the action potential are stabilized by barium against the displacement of the membrane potential shows that the inactivation of the sodium-carrying system normally occurring upon depolarization does not take place. In other words, under the influence of barium, the sodium-carrying system is largely stabilized at a half depressed state, so that its rapid availability for carrying sodium ions upon depolarization is reduced, thereby slowing down the rate of rise of the action potential, and so that after activation its inactivation proceeds very slowly, contributing towards slowing down the rate of fall of the action potential. The fact that the spike height is not reduced by barium does not seem to be inconsistent with this view, because the delay of the inactivation occurring upon depolarization together with the decrease in potassium conductance rise, which will be discussed later, would enable the membrane to be depolarized as far as or even beyond the normal level.

The increase in negative after-potential caused by barium cannot be explained in terms of the accumulation of any substance near the membrane, because no linear addition of the negative after-potentials occurs during repetitive excitation. In barium the membrane resistance is considerably higher than normal not only in the resting state but also in the depolarized membrane. This leads us to the assumption that the potassium conductance, which rises with a certain delay upon depolarization, is still less than normal at any particular level of depolarization. It follows that the potassium conductance during the falling phase of the action potential is smaller than normal, thereby preventing the falling phase from reaching the resting level at once, forming a large negative after-potential. The depression of the inactivation process normally occurring upon depolarization, which has been discussed in the preceding paragraph, is also likely to be partly responsible for the large negative after-potential. The positive phase following the spike, which is thought to be indicative of the increase in potassium conductance (Yamasaki & Narahashi, 1959*a*), disappears in barium, this effect being also well explained in terms of a smaller potassium conductance.

The falling phase of the catelectrotonic potential has a time constant appreciably smaller than that of the resting membrane. Furthermore, the rate of the falling phase depends partly on the magnitude of depolarization, becoming slightly higher with increasing depolarization. It follows that some accelerating mechanism for the falling phase comes into action when the membrane is depolarized. It is reasonable to suppose that this accelerating mechanism is a rise in potassium conductance. On the other hand, the time constant of the falling phase of the negative after-potential is approximately the same as that of the catelectrotonic potential and

appreciably smaller than that of the resting membrane. This is explained on the assumption that the accelerating mechanism, i.e. the rise in potassium conductance, is still working slightly during the falling phase of the negative after-potential, although the mechanism is now partly suppressed by barium, as mentioned above.

It is worth while to note in this connexion that in barium the falling phase of the negative after-potential is slightly but definitely accelerated upon repetitive stimulation, whereas the reverse is true in normal Ringer's solution (Narahashi & Yamasaki, 1960*a*). This acceleration may be explained as follows. For the first impulse the potassium conductance during the falling phase is still less than normal. By repetitive excitation however, a small additional increase in potassium conductance may occur. Such an increase would accelerate the falling phase of the negative after-potential, thereby making the time constant smaller.

SUMMARY

1. The effect of Ba on the spike potential and the negative after-potential of the cockroach giant axon has been examined by means of intracellular micro-electrodes.

2. Nerve conduction was reversibly blocked by isotonic Ba solution and a solution with 75 mM-Na and 93 mM-Ba.

3. By 21–76 mM-Ba the resting potential was slightly reduced, the rising and falling phases of the action potential were considerably slowed down, and the negative after-potential was markedly increased, the degree of the effects being dependent on the Ba concentration. The spike amplitude slightly increased in weak concentrations of Ba.

4. The critical depolarization for firing became greater but the threshold current remained almost unaltered in 43 mM-Ba.

5. The height and the rate of rise of the action potential were stabilized by 43 mM-Ba, undergoing smaller changes on displacement of the membrane potentials.

6. The negative after-potentials increased by 43 mM-Ba did not add linearly during repetitive excitation.

7. The falling phase of the negative after-potential in 43 mM-Ba could be expressed by two exponential terms. The initial phase, occupying the major part of a negative after-potential, was slower than the terminal phase. Both phases were slightly accelerated by repetitive stimulation.

8. In 43 mM-Ba, the time constants for the falling phase of the negative after-potential and of the catelectrotonic potential were appreciably smaller than the time constant of the resting membrane.

9. The effective membrane resistance was remarkably increased by 43 mM-Ba not only in the resting state but also in the depolarized membrane.

10. In isotonic Ba, neither spikes nor local responses were produced by transmembrane stimulation.

11. The Na-carrying system is to some extent stabilized by Ba at a half depressed state. The increase in negative after-potential caused by Ba can be explained on the assumption that the rise in K conductance during the falling phase of the action potential falls short of the normal level and that the inactivation normally occurring upon depolarization is much depressed, thereby preventing the falling phase from reaching the resting potential at once.

I am indebted to Dr T. Yamasaki for his interest and encouragement during the course of this work. Thanks are also due to Dr S. Hagiwara and Dr A. Watanabe for criticisms, and to Miss S. Hamada for technical assistance. This work was supported in part by a grant from the Ministry of Education of Japan.

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PROCEEDINGS OF
THE PHYSIOLOGICAL SOCIETY

NATIONAL HOSPITAL MEETING, LONDON

17–18 *February* 1961

Electro-nystagmographic equipment for the investigation in the human subject of spontaneous nystagmus and certain other oculomotor abnormalities

By C. S. Hallpike, J. D. Hood and E. Trinder. *Medical Research Council Otological Research Unit, National Hospital, Queen Square, London, W.C.* 1

Equipment for the micro-dissection of the inner ear in small laboratory animals

By L. Citron and C. S. Hallpike. *Medical Research Council, Otological Research Unit, National Hospital, Queen Square, London, W.C.* 1

Equipment for the rapid microscopic examination of serial sections

By C. S. Hallpike, D. Piper and J. L. Tudman. *Medical Research Council Otological Research Unit, National Hospital, Queen Square, London, W.C.* 1, *and Engineering Division N.I.M.R.*

Equipment for human temporal bone microtomy

By J. C. Billingham and S. J. Gray. *Medical Research Council Otological Research Unit, National Hospital, Queen Square, London, W.C.* 1

A transistor-stabilized low-tension supply

By G. D. Dawson and J. R. Pitman. *The Medical Research Council Neurological Research Unit, The National Hospital, Queen Square, London, W.C.* 1

The power unit to be demonstrated has been designed to supply the heater current at 12 V for the earlier stages of valve amplifiers. When a valve amplifier is first connected to an accumulator it commonly draws 5 or 10 times more than the normal running current until the valve heaters have warmed up. In a stabilized supply the regulating transistors must

therefore either be capable of handling this 10 times overload, which is usually uneconomical, or some form of overload protection must be provided. Fuses, or other devices which disconnect the supply, are clearly unsuitable (Kemhadjian & Newell, 1959). The unit to be demonstrated has a very low output impedance when supplying currents up to 3 A, but at higher currents the output impedance is arranged to rise rapidly and on short circuit the current limits at about 4·5 A. This prolongs the heating time of the valves, but not seriously, and the initially low resistance of the cold heaters does not harm the unit.

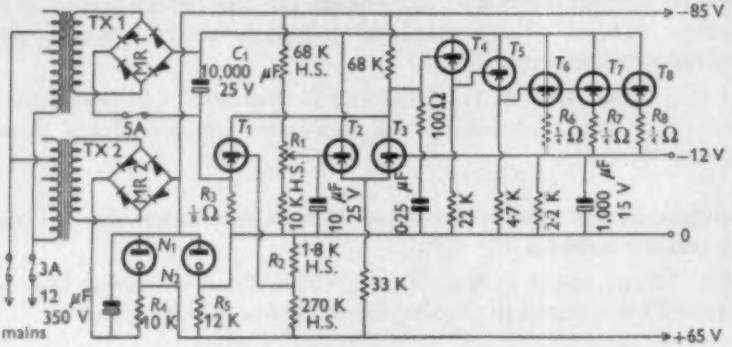

Fig. 1. Circuit of 12 V, 3 A supply. TX 1 is a 12·6 V, 5 A transformer and TX 2 a 250 V, 25 mA transformer. MR 1 and MR 2 are selenium bridge rectifiers rated at 15 V (R.M.S.), 4 A and 250 V (R.M.S.), 25 mA respectively. T_1–T_4 are Mullard transistors type OC 71 and T_5–T_8 Newmarket transistors type V 30/30P. N_1 (left) is a Mullard stabilizing tube type 150 B2 and N_2 (right) a Mullard reference tube type 85 A 2. R_2, the output voltage control, is a 5 kΩ wire-bound potentiometer. R_3 is a length of constantan wire. ('K' in figure = kΩ).

The main part of the circuit is conventional. The output voltage is controlled by the transistors T_6, T_7 and T_8, in parallel, which are mounted directly on a heat sink of 50 sq.in. (323 cm²) of 16 S.W.G. aluminium which is blackened and insulated from the chassis. The resistors R_6, R_7 and R_8 in the emitter circuits of these transistors help to equalize the currents through them and are made from suitable lengths of enamelled copper wire wound on formers. The transistors T_4 and T_5 are current amplifiers to provide a low impedance source for the base currents of the regulators T_6–T_8 from the high impedance at the collector of T_3. The pair of transistors T_2 and T_3 form a difference amplifier with the common emitter load of 33 kΩ taken to the reference line of +65 V. The output voltage is connected to the base of T_2 and the reference voltage, which is derived from the −85 V line through R_1, is applied to the base of T_3. The voltage changes at the collector of T_3, which are an amplified version of changes of the output

voltage with reference to the base voltage of T_2, are applied to T_4 in such a sense that they oppose a change of output voltage. The transistor T_1 plays no part in the operator of the circuit at load currents up to 3 A, as it is biased to cut-off by the positive potential applied to its base. When the output current approaches 4·5 A the voltage drop across the resistor R_3 exceeds the positive cut-off voltage on the bases of T_1. T_1 therefore begins to conduct and pulls down the collector of T_3, reducing the output voltage of the supply sufficiently to maintain the current at 4·5 A, at which level the output transistors T_6–T_8 remain inside their safe rating.

The output impedance of the stabilizer at 3 A is 0·01 Ω and it reduces a change of mains input voltage of 10% to an output change of 0·05%. The maximum noise and ripple on the output at full load is 1 mV.

The power transistor T_5, which is mounted on the same heat sink as T_6–T_8, is capable of driving additional regulating transistors if a greater current than 3 A is required. In this case the number of regulating transistors, the size of the heat sink on which they are mounted and the capacity of the reservoir capacitor C_1 must be increased proportionately. The value of R_2, which controls the positive cut-off bias on the base of T_1, should also be increased so that the limiting output current is approximately 50% more than the normal load current required. Minor modifications only are required for an output of 6 V. The reference voltages of +65 and −85 are derived from a separate transformer and rectifier, the output of which is stabilized by the neons N_1 and N_2. They may be used as references for other stabilizers provided R_4 and R_5 are adjusted to keep the currents through the neons at the maker's rating. In the demonstration the supply will be shown in conjunction with two others providing −6 V and +6 V which supply power for transistorized apparatus.

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A transistor-stabilized high-tension supply

By G. D. DAWSON and J. R. PITMAN. *The Medical Research Council Neurological Research Unit, The National Hospital, Queen Square, London, W.C.* 1

This unit will supply 250 V at 200 mA for operating valve circuits. Because of the high efficiency of the silicon rectifiers used for the power supply and of the transistors which are used for the regulating elements, the bulk of the unit is reduced, and the heat output greatly reduced, in comparison with units using valve rectifiers and regulators. Two of these units will be demonstrated mounted on a single panel 19 in. × 5·25 in. (48·3 × 13·3 cm) in a chassis 12 in. (30·5 cm) deep. Either the negative or

positive side of the output may be earthed to provide positive or negative supplies.

The circuit is basically a series stabilizer using neons N_1 and N_2 to obtain the reference voltage. In order to allow for changes in the rectifier output with load current changes, and with changes of mains voltage, the series elements of the regulator must be able to withstand about 100 V, nearly half the output voltage of the supply. To meet this requirement two easily available and relatively inexpensive 60 V transistors (T_7 and T_8) are used in series, so arranged that the voltage across them both is shared equally

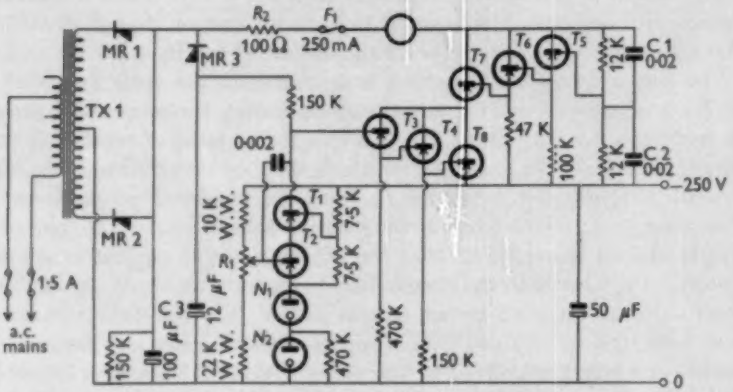

Fig. 1. Circuit of 250 V, 200 mA supply. TX 1 is a 250, 0, 250 V R.M.S. 250 mA, transformer which should have a resistance of $< 20\,\Omega$ for each half of the secondary. MR 1, 2 and 3 are 250 V R.M.S. silicon rectifiers (Radiospares type REC 51). N_1 and N_2 are Mullard reference tubes type 85 A 2. T_1 to T_8 are transistors type V 60/20 IP and T_7 and T_8 type V 60/30 P (Newmarket). R_1 is a 5 kΩ wire-wound potentiometer and controls the output voltage. The current meter (circle to right of F_1) has a full-scale deflexion of 250 mA. T_4 and T_8 are mounted on one sheet of 40 sq.in. (258 cm²) of aluminium which is blackened and insulated from earth and T_6 and T_7 on another similar sheet. T_1 and T_2 and T_3 and T_5 do not require a heat sink and may be mounted on a tag strip. All electrolytic capacitors are suitable for working at 350 V. ('K' in figure = kΩ).

between them. For the same reason two transistors in series (T_1 and T_2) are used to compare the output voltage with the reference voltage and amplify the difference. The amplified difference voltage at the collector of T_1 is taken to the current amplifiers T_3 and T_4 which drive the base of T_8 in such a way that changes of output voltage with load current or input voltages are opposed. T_5 and T_6 provide the current amplification to drive the base of T_7 from the two 12 kΩ resistors in series which form a centre tap across the transistors T_7 and T_8.

The maximum voltage rating of the transistors must not be exceeded

even instantaneously. The circuit is so arranged that this condition is met both during the period immediately after switching on and in the event of overload or complete short circuit. Quick blowing fuses will not prevent a momentary voltage overload which would be sufficient to destroy the transistors. Protection is achieved in the following way. If the output is short-circuited the transistors T_1 and T_2 cease to conduct. The rectifier MR 3 prevents the capacitor C_3 from discharging so that a current continues to flow into the base of T_3. The high current amplification following T_2 causes a current of 2 A or 3 A to flow through T_7 and T_8. This current flowing through the 100 Ω resistor R_2 is sufficient to reduce the voltage across T_7 and T_8 almost to zero until the fuse F_1 has time to blow. The capacitors C_1 and C_2 prevent any rise of voltage across T_7 and T_8 which might occur at the instant of short circuiting because of limited high-frequency response of the circuit. When switching on, the rectifier MR 3 allows the capacitor C_3 to charge up quickly. If a resistor were used here instead of a rectifier it would prevent C_3 from discharging quickly on short circuit, but it would also prevent it charging up quickly and therefore T_2 would be cut off for a short period after switching on, and the voltage rating of T_7 and T_8 would be exceeded.

The d.c. output resistance of the unit is $> 2\Omega$ over the current range of 0–200 mA and peak currents of 250 mA may be drawn. The maximum noise and ripple on the output is 20 mV and the stabilization ratio is such that a 10% change in mains input voltage produces $> 0{\cdot}2\%$ change in output volts.

Inhibition of muscular activity in the lightly anaesthetized cat induced by cortical stimulation and recorded by electromyograms

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Stereotaxic operations on patients with disorders of the motor system have given the opportunity to stimulate in the conscious subject the internal capsule, thalamus and basal ganglia, while recording the peripheral effects with electromyography. In about one quarter of 115 patients with the chronic rigidity and tremor of Parkinsonism, stimulation has caused a well-localized temporary cessation of muscular activity in parts of the contralateral limbs. Although the site of stimulation is known precisely in relation to the wall of the 3rd ventricle, the anatomical location of the electrode cannot be stated with certainty. It is, however, likely that it is in the internal capsule, and that the response observed is similar to that reported by a number of workers to follow stimulation of the motor region of the cortex in patients with this particular condition.

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This encouraged us to re-examine in lower animals the inhibitory phenomenon that can be produced by cortical stimulation. Denny-Brown & Botterell (1948) observed that 'melting of the posture' in the contralateral arm of a Rhesus monkey, suspended and lightly anaesthetized with ether, could be provoked by stimulation of a region immediately forward of the precentral gyrus. We have developed a technique for maintaining a cat in a state of light anaesthesia using N_2O, O_2 and Fluothane (I.C.I.) with endotracheal intubation. If the animal is suspended in this state the free hanging limbs show tonic and phasic activity of various types although the animal is unconscious (absent cough-reflex). Electromyograms from four main muscle groups in the four limbs are obtained by indwelling bipolar electrodes made from twisted enamelled stainless-steel wire (0·2 mm diam.), and recorded on sixteen ink pens. Cortical stimulation is performed with a 1 mm concentric electrode and the electrocortigram (e.co.g.) is recorded from the neighbouring cortex to detect after discharge or spreading depression if they should occur. Under these conditions stimulation of the peri-cruciate region can cause temporary localized cessation or diminution of muscular activity. This effect is apparently distinct from that related to reciprocal innervation of antagonists; it may be ipsilateral as well as contralateral and is not accompanied by changes in the e.co.g.

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A method for the measurement of sweat output of the human forearm.

By A. H. G. LOVE and R. G. SHANKS. *Department of Physiology, The Queen's University, Belfast*

The cerebral soluble proteins of cerebral oedema

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Enzymic activity of normal and dystrophic muscle

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Normal and abnormal muscle protein patterns

By B. P. HUGHES. *Department of Chemical Pathology, Institute of Neurology, The National Hospital, Queen Square, London, W.C.* 1

Conduction delays and the judgment of simultaneity

By A. M. Halliday and Rosemary Mingay. *Medical Research Council Neurological Research Unit, The National Hospital, Queen Square, London, W.C.* 1

Comparative analysis of the cerebral arterial system in laboratory animals and man

By D. Tompsett and B. Wyke. *Royal College of Surgeons of England, London, W.C.* 2

Changes in the human retinogram during compression of the eye

By W. A. Cobb and H. B. Morton. *Department of Applied Electrophysiology, The National Hospital, Queen Square, London, W.C.* 1

Motor and sensory conduction in the lateral popliteal nerve of man

By R. W. Gilliatt and R. G. Willison. *Department of Applied Electrophysiology, The National Hospital, Queen Square, London, W.C.* 1

Some recent work on the electro-nystagmographic analysis of spontaneous nystagmus and other oculomotor abnormalities

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Modification of thalamic transmission by sensory stimulation

By A. Angel* and G. D. Dawson†. *Department of Physiology, University College London*

Dawson, Podachin & Schatz (1959) showed that in rats, at some levels of trichloroethylene anaesthesia, parts of the electrical response recorded in the primary cortical receiving area after stimulating a forepaw may become enlarged if any part of the body surface has been stimulated beforehand by pinching, rubbing or by trains of electrical stimuli. This enlargement was not accompanied by an increase in the electrical response in the cuneate nucleus. Thus it seems that the change was due either to an increase in size of the afferent volley above the cuneate nucleus, or to a change in state of the cortex. Transmission through the thalamus has

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been studied in rats anaesthetized with urethane, by recording the electrical responses either from a mass of thalamic neurones or, extracellularly, from single neurones.

During the mass response the thalamus first becomes positive with respect to other parts. This potential change has a duration of 1 msec and is relatively resistant to asphyxia. The second part of the response is negative-going, is readily abolished by asphyxia and has a duration of 2–5 msec. Other waves may follow these two. The first wave is taken to indicate the arrival of the afferent volley and the second to indicate the discharge of post-synaptic elements. The group of cells studied responded only to stimuli on the contralateral body surface, and commonly only to stimulation of one limb. If a response was obtained from both fore and hind limbs it was usually much greater from one than from the other. In some instances a response could be obtained only from stimulation of a single small area, and this was so for all the single neurones studied. For this reason it seems likely that the part of the thalamus and the single neurones studied were in the direct cortical sensory pathway. After conditioning which caused the increase in the cortical responses it was found that the thalamic responses became more stereotyped, the scatter in their latency was less and a second negative wave often appeared in the mass response. The latency of discharge of single neurones was decreased and became less scattered, and the probability that they would fire was increased. When the conditioning stimuli were applied to the same side of the body as that of the thalamus from which records were being made they did not fire any of these cells in the direct sensory pathway.

Apparently the increase in the cortical response could be partly explained by the increased transmission through the thalamus. These results do not exclude the possibility that the conditioning stimuli were altering the state of the cortex, and they do not indicate the pathways through which the conditioning stimuli operate.

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The development of acetylcholine sensitivity in nerve-free muscle segments

By B. Katz and R. Miledi. *Department of Biophysics, University College London*

The sensitivity of a muscle fibre to ACh increases after denervation and gradually spreads from the motor end-plate over the rest of the fibre (Ginetsinskii & Shamarina, 1942; Axelsson & Thesleff, 1959; Miledi, 1960).

This process might be thought to originate from some chemical activity at the end-plate zone, and it seemed worth inquiring therefore whether a severed portion of a muscle fibre which contains no nerve endings would be able to develop ACh sensitivity similarly to an intact fibre after denervation.

Sartorii of frogs, operated under ether, were cut so that the aneural pelvic portion (approx. one quarter) was separated from the remainder of the muscle. Intracellular potential measurements were made on the isolated muscles at varying times after the operation, and their ACh sensitivity was tested by localized iontophoretic application with a micropipette. After a few days, many fibre segments showed visible signs of damage and had very low resting potentials all along, but there was good recovery in a large proportion within less than two weeks.

The following points of interest arose: (*a*) The aneural segment became highly sensitive to ACh within about 2 weeks (20° C), much faster and more strikingly than is found in frog muscle after ordinary denervation. ACh sensitivities measured in the nerve-free segments of many fibres were not far below those of normal end-plate foci (cf. Miledi, 1960). (*b*) The increase in sensitivity must be attributed, at least partly, to after-effects of injury (and subsequent self-repair) rather than simply to removal of the nerve influence, for a considerable spread of ACh sensitivity also occurred in the innervated (and transmitting) tibial three-quarters of the muscle fibres.

The experiments show: (i) that a part of the muscle fibre which is remote from the end-plate zone can independently develop a high drug sensitivity; (ii) that there are ways of inducing supersensitivity (in the present case by muscle injury) even when the functional connexions with the motor nerve have been maintained.

A further observation made in the course of these experiments was that some fibres of the control muscles (undivided) showed, apart from the high ACh sensitivity at the end-plate focus, a detectable sensitivity localized at the muscle–tendon junction. This is possibly related to the similar localization of ACh esterase (Couteaux, 1953; Gerebtzoff, 1954).

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The mitral cells of the rabbit's olfactory bulb

By C. G. PHILLIPS, T. P. S. POWELL and G. M. SHEPHERD.* *Departments of Physiology and Human Anatomy, University of Oxford*

For an understanding of transmission through the olfactory bulb, a knowledge of the properties of the mitral cells is of primary interest. In rabbits under urethane-chloralose anaesthesia, we have identified the mitral cells physiologically by recording, with fine extracellular micro-electrodes, their responses to antidromic stimulation of their axons in the lateral olfactory tract.

As the micro-electrode advances from the surface of the bulb to the periventricular layer, while shocks are delivered to the tract, a succession of potential waves is recorded. Specific wave patterns have been found, by combined histological and experimental measurement, to be regularly associated with the successive layers of the bulb (Cajal, 1911). Throughout the bulb an early negative wave, latency about 1·5–2·5 msec and duration about 1·5 msec, is recorded. With increasing depth its latency decreases and amplitude increases. From the surface to the outer plexiform layer it is preceded by a small positive wave, and in the outer plexiform layer it is followed by a second negative wave. Deep to the layer of mitral cell bodies a large positive wave replaces the second negative wave. Thus, when unitary spikes are encountered, the associated waves serve as a guide to the layer in which the electrode tip is located.

The majority of unit spikes with short, unvarying latency and the ability to follow high rates of tract stimulation—properties suggesting antidromic excitation—were found at or near the layer of mitral cell bodies. These extracellular positive-negative spikes sometimes reached 'giant' size (Granit & Phillips, 1956), up to 55 mV peak-to-peak. The spikes usually showed a discontinuity on the positive-going upstroke, as in cerebellar Purkinje cell spikes (Granit & Phillips, 1956). This initial component of the impulse could be fired in isolation by closely spaced (paired or repetitive) tract shocks, as in cortical pyramidal cells (Phillips, 1959). Presumably the antidromic mitral cell impulse meets two successive regions of excitable membrane, as in the spinal motoneurone (Brock, Coombs & Eccles, 1953).

Smaller unitary responses were recorded in the outer plexiform later. Their latency was longer and was variable, but they always fired singly to each tract shock. They are possibly tufted cells, as this is the region in which their cell bodies occur. In the granular layer, deep to the layer of mitral cell bodies, we recorded small spikes of variable latency, some of which responded repetitively to each shock. The number of impulses was

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sometimes related to shock strength. These are probably impulses in granule cells or Golgi II cells, excited synaptically. The source of such synaptic excitation (direct or relayed) could be recurrent collaterals of the mitral axons or centrifugal fibres (Cajal, 1911) in the olfactory tract.

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The immediate effects of expiratory obstruction on breathing

By E. J. M. Campbell, C. J. Dickinson, O. P. Dinnick and J. B. L. Howell. *Departments of Medicine, Anaesthetics and Pharmacology, and the Institute of Clinical Research, The Middlesex Hospital Medical School, London, W.*1

A 'threshold' load, that is, an opposition of a constant pressure, was added to the expiratory phase of respiration of conscious and anaesthetized subjects breathing in a closed spirometer circuit. This was achieved by switching the expiratory gas flow path through a tube passing down into water in a bottle.

The first effect of such a load was partially or completely to prevent expiration, depending on the opposing pressure. There was no apnoea and the frequency of breathing was unaffected. The first inspiration following the addition of the load was to an increased lung volume implying the development of greater tension by the inspiratory muscles. This increase persisted as long as the load was present and disappeared immediately it was removed. The speed of this response suggests a nervous reflex.

When expiration was partially obstructed in the same way in the dog, inspiration was also to a greater lung volume but this response was obscured by apnoea and slow breathing (the Hering–Breuer reflex). Bilateral vagotomy caused slow deep breathing and expiratory loading no longer caused apnoea. In most dogs expiratory loading also no longer caused an increased end-inspiratory lung volume.

Rhythmic stimulation of the central ends of the cut vagi reduced the depth of breathing. Expiratory loading then caused an increased end-inspiratory lung volume with no apnoea, that is, a response like that seen in man.

Evidence will be presented that the increased force of inspiratory contraction caused by expiratory obstruction is due to the increased elastic load the distended thorax imposes on the inspiratory muscles.

The mechanism may therefore be the same as that previously discussed (Campbell, Dickinson, Dinnick & Howell, 1961).

These experiments: (i) confirm that adult man has a weak Hering–Breuer reflex; (ii) demonstrate that there is a nervous mechanism which increases the end-inspiratory tension developed by the inspiratory muscles when expiration is obstructed; (iii) provide another example of the rapid stabilization of tidal volume when the mechanical conditions of breathing are altered.

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Occurrence of amine oxidase in an insect, *Periplaneta americana* L.

By H. BLASCHKO, E. H. COLHOUN and NORA FRONTALI. *Department of Pharmacology, University of Oxford*

Amine oxidase has been found in a number of different invertebrate phyla: Molluscs, Annelids and Echinoderms. The experiments to be described show that the enzyme occurs also in a member of the Arthropoda.

Homogenates of different organs of the male cockroach, *Periplaneta americana* L., were prepared in 0·067 M sodium phosphate buffer of pH 7·4 and incubated with various amines at 30 °C in an atmosphere of oxygen.

In the manometric experiments only one preparation gave significant oxygen uptakes; this was a homogenate of the Malpighian tubules. From the initial rates of oxidation the activities, expressed as μl. O_2/g fresh tissue/hr, were: with *iso*amylamine 390, with β-phenylethylamine 416; with N-methyl-β-phenylethylamine 181; with tryptamine 100; with 5-HT the uptake of oxygen was not significant.

These activities are of an order similar to that found in mammalian liver. In some of the other tissues the presence of amine oxidase could be demonstrated by a sensitive qualitative test: the characteristic smell of the aldehydes formed in the enzymic reaction from either β-phenylethylamine (see Florence & Schapira, 1945) or *iso*amylamine. Amine oxidase was found in the whole head, flight muscles, gastric caeca, mid- and hind-gut and fat body.

These observations are of interest in view of reports that catechol amines and 5-HT occur in insects (Cameron, 1953; Östlund, 1954; Colhoun, 1959; Jaques & Schachter, 1954).

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Single cortical unit activity evoked by splanchnic stimulation

By P. P. NEWMAN. *Department of Physiology, School of Medicine, Leeds*

Amassian (1951) and Downman (1951) recorded surface primary responses in the somatosensory areas following stimulation of a splanchnic nerve. The present communication describes the pattern of activity evoked in single cortical neurones of somatosensory areas I and II in cats anaesthetized with thiopental sodium (50 mg/kg) administered intraperitoneally. The animals were maintained under light anaesthesia and given intravenous flaxedil. The left splanchnic nerve was stimulated by a pair

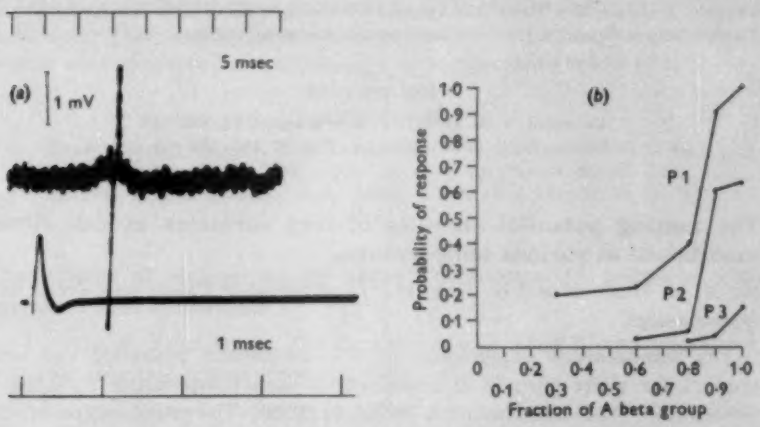

Fig. 1. (*a*) Single unit response to stimulation of contralateral splanchnic nerve. Upper record, positive-negative spike occurring about the peak of reversed primary potential. Latency 14·9 msec. Lower record, A beta fraction of splanchnic nerve action potential. Time interval, upper beam 5 msec, lower beam 1 msec. (*b*) Three curves showing the effect of increasing the A beta fraction on probability (P_1, P_2, P_3) that at least 1, 2 or 3 discharges occur per sweep.

of silver electrodes and the nerve action potential recorded from a proximal pair. Recordings from the contralateral cortex were made through glass micro-electrodes filled with 3M-NaCl or 3M-KCl (tip diameter 0·5–3·0 μ; resistance 15 – 8 MΩ).

Under light anaesthesia, the characteristic unit response to splanchnic stimulation was a diphasic positive-negative spike 3–5 mV in amplitude. In different units the mean latency ranged between 11·4 and 18·5 msec. The threshold of the cortical response was determined in terms of the fraction of A beta fibres excited.

Increasing the stimulus strength above threshold tended to shorten the latency of the response. In one intensity series, when the fraction of A beta fibres excited was increased from 25 to 55 %, the mean latency of the

unit response was reduced from 16·2 to 14·3 msec. In some units increasing the strength of stimulation above the threshold usually increased the number of spikes discharged. Increasing the stimulus strength generally increased the probability that the unit would fire at least one spike. Many cortical units responded with a high probability of discharge to excitation of the A beta group. Other units produced a discharge at a low probability with A beta excitation alone, but could be activated with a high probability of discharge by stimulating in addition the A gamma-delta groups.

This investigation was carried out in the Department of Physiology, Albert Einstein College of Medicine, New York. It is a pleasure to thank Professor V. E. Amassian for much generous help in developing the technique and in discussing the data.

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The resting potential stability of frog sartorius muscle fibres maintained at various temperatures

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The temperature dependence of the membrane potential has been studied for short periods of exposure to each temperature by Ling & Woodbury (1949) and Nastuk & Hodgkin (1950). The present experiments were carried out to determine the behaviour of the resting potential when the exposure to each test temperature was maintained for 4 hr followed by a return to the reference temperature (14° C) for a further 2 hr. The mean resting potential (R.P.) was determined from a sample of 20 fibres as previously described (Draper, Friebel & Karzel, 1959). Paired sartorius muscles from *Rana temporaria* were used, one was maintained at 14° C as a control. The mean R.P. at 14° was 90·3 mV (S.E. $\pm$ 0·08, 280 observations). At 3·5° C the mean R.P. was 87·2 mV $\pm$ 0·18 (n = 20) and at 25° C 93·4 $\pm$ 0·49 (n = 20). The R.P. had changed to its new value as soon as observations could be made (6–10 min after the change), and below 20° C the mean R.P. remained constant for 4 hr and returned to control values promptly when replaced in Ringer's solution at the reference temperature. At 20 ° or higher the resting potential was maintained for a progressively shorter time, 2 hr at 20° C and a few minutes at 38° C. Above 20° C the subsequent fall in R.P. was profound and irreversible. The very rapid decline in R.P. at 38° C was associated with the onset of an irreversible contracture of the muscle.

At temperatures of -1 to $+1°$ C the initial fall in R.P. was larger than expected (84 mV) and the R.P. steadily decreased during the 4 hr, reaching a final value of about 77 mV. Subsequent rewarming was followed by a slow return to control values. Between the temperatures of 5 and 20° C the variation of the resting potential with the absolute temperature has a slope in agreement with expectations based on the Nernst equation. Below 5° C the slope increases and above 20° there is a maximum at 25° C for short exposures. The maximum value shifts to lower temperatures with an increase in time of exposure and lies between 15 and 20° C for a 4 hr exposure.

Action potential studies have confirmed the results obtained by Nastuk & Hodgkin (1950) with the addition point that the action potential duration is very sensitive to small changes in temperature below 0° C.

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The effects of ischaemia on nerve conduction in patients with carpal tunnel syndrome

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The carpal tunnel syndrome is caused by compression of the median nerve at the wrist under the flexor retinaculum, and it is characterized by attacks of pain and paraesthesiae in the hand occurring particularly at night. These fluctuating symptoms have been attributed to intermittent ischaemia of the nerve fibres under the flexor retinaculum. Experiments were undertaken to study the effects of acute ischaemia on the motor fibres of the median nerve.

The median nerve was stimulated at the wrist by a brief shock which was supramaximal for motor fibres and the muscle response recorded from the thenar muscles with surface electrodes. The amplitude and the area of the evoked muscle action potentials were then measured from photographic records. Ischaemia was produced by inflating a pneumatic cuff round the arm to above arterial pressure and maintained for periods of up to 30 min.

When control experiments were performed on healthy subjects it was found that ischaemia for 20 min produced no significant change in the muscle action potential, and that after 30 min action potential amplitude and area had not fallen to less than 50% of their initial value.

In patients with the carpal tunnel syndrome tested in the same way the effect of ischaemia was variable. Whereas some patients were not more susceptible to ischaemia than control subjects there were others in whom the action potential was reduced to less than 25% of its initial size within 20 min. In 6 out of the 10 patients action potential area and amplitude had fallen to less than 50% of their initial value by the end of 30 min. This effect of ischaemia was not due to venous congestion; nor was it due to a rise of threshold under the stimulating electrodes at the wrist, as similar results were obtained when the stimulus was applied to the median nerve at the elbow.

Susceptibility to ischaemia in patients with the carpal tunnel syndrome did not appear to be related to the severity of the pre-existing nerve damage as judged by either the initial size or initial latency of the muscle action potential in response to nerve stimulation. It was, however, related to the severity of the patients' spontaneous attacks of pain and paraesthesiae in the period immediately preceding the test.

The membrane potential and mechanical response of smooth muscle cells of the rabbit colon to stimulation of the extrinsic sympathetic nerves

By J. S. GILLESPIE*. *Rockefeller Institute, New York, U.S.A.*

The spontaneous electrical and mechanical activity of smooth-muscle cells of the rabbit colon and their response to stimulation of the extrinsic parasympathetic fibres has been previously reported (Gillespie, 1960). The present paper describes the response to stimulation of the extrinsic sympathetic nerves. The double-innervated rabbit colon preparation was used (Garry & Gillespie, 1955). The tension of the longitudinal muscle layer was recorded isometrically with an R.C.A. 5734 electro-mechanical transducer valve and the membrane potential of single smooth-muscle cells recorded with an intracellular micropipette. The sympathetic nerves were stimulated electrically under a layer of liquid paraffin.

The membrane potential of the smooth-muscle cells is unstable (Gillespie, 1961). Regular, slow waves of potential charge lasting several seconds are seen, the depolarization phase of which culminates in a brief burst of spike potentials followed by repolarization. Associated with each burst of spike potentials is a contraction of the longitudinal muscle. Stretching the preparation prolongs these periods of spike activity, and eventually the discharge of spike potentials becomes continuous at an average frequency of 1/sec. Single stimuli to the sympathetic nerves are

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ineffective in the spontaneously active preparation. Single stimuli to the sympathetic nerves preceding single stimuli to the parasympathetic nerves by a varying interval are similarly ineffective in modifying the mechanical and electrical response to the latter. Repetitive stimulation at frequencies greater than about 10/sec produce complete mechanical inhibition. The membrane potential rises to the maximum observed during the slow potential waves and remains there for the duration of stimulation; both slow waves and spike potentials are suppressed. There is no hyperpolarization. At frequencies of 10 sec and less mechanical inhibition is commonly incomplete. Individual smooth-muscle cells continue to discharge spike potentials but the rate of depolarization during the slow waves is reduced so that the interval between spikes is prolonged. A new phenomenon is also seen; dissociation between mechanical and electrical activity. Contractions occur with no associated spike potentials and vice versa. In preparations stretched so as to produce a continuous discharge of spike potentials stimulation of the sympathetic nerves at frequencies above 10/sec produces hyperpolarization as well as suppression of slow waves and spike potentials.

A hypothesis which would explain these results is that the smooth-muscle membrane is sensitive to mechanical deformation and is depolarized by stretch. The sympathetic transmitter stabilizes the membrane potential, abolishing the slow waves and the spikes arising from them and in the stretched (depolarized) preparation causing in addition actual hyperpolarization. If stabilization of the membrane interferes with the process of conduction between cells before it suppresses all spontaneous activity in individual cells, as suggested by Bozler (1940), this would explain the dissociation of mechanical and electrical events at low frequencies of nerve stimulation.

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A tachistoscopic study of visual inattention

By M. Kinsbourne and Elizabeth Warrington. *The National Hospital, Queen Square, London, W.C.* 1

Patients showing visual inattention to confrontation on bilateral simultaneous stimulation were tested tachistoscopically. It was possible in some of them to reproduce the phenomenon under those conditions. The method permitted the measurement of thresholds in terms of length of exposure time of the stimulus; stimuli could be presented simultaneously or successively at various time intervals.

In some cases the thresholds in the intact and impaired fields of vision were shown to be the same. Inattention in those cases cannot be explained in terms of visual threshold deficits.

Where the threshold in the impaired half field is relatively raised, then this was corrected for by suitable adjustment of the duration of the two exposure times. In some cases the phenomenon of inattention still persisted and was thus shown to be independent of the visual threshold deficit. In other cases, inattention was no longer obtained and must therefore have been a function of the threshold alteration.

It was concluded that visual inattention is not a function of visual threshold changes, but can nevertheless be mimicked where there is a relative failure in one half-field.

The effect of successive posterior parietal lobe ablations on tactile shape discrimination in the monkey

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A clear functional relationship has been established between the sensorimotor cortex of one cerebral hemisphere and the limbs on the opposite side of the body. Little such information is available for the posterior parietal areas (formerly known as areas 5 and 7). It is, however, known that one-stage bilateral ablation of these posterior parietal areas gives rise to a variety of disabilities in the monkey (Bates & Ettlinger, 1960). One of these is an impairment of tactile shape discrimination. In the present experiment the effect of successive unilateral posterior parietal ablations in the monkey has been investigated.

Thirteen animals were trained to distinguish between two shapes by touch in the dark. The positive shape was a solid 'L', the negative shape was a 'T'. The right hand was restrained during training, so that the discrimination was learnt by palpation with the left hand of all animals. Eight of these animals then received successive posterior parietal ablations with training in between operations. The remainder received other parietal ablations.

Four animals that received a left-sided (ipsilateral) posterior parietal ablation as their first lesion were little if at all impaired at tactile shape discrimination with the left hand in the dark. Four animals receiving a right-sided (contralateral) ablation first were moderately impaired. These latter four animals gave evidence of little if any additional impairment when they received a left-sided ablation subsequent to their first (contra-

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lateral) lesion. The other four animals were again only moderately impaired (that is, no more severely than animals receiving a contralateral ablation as their only lesion) when they received a right-sided ablation subsequent to their first (ipsilateral) lesion. These findings suggest virtual independence of left and right posterior parietal areas for tactile discrimination.

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An investigation of the equivalence between auditory and visual rhythms in man

By S. L. Chorover* and M. Cole†. *The Psychological Laboratory, Institute of Neurology, The National Hospital, Queen Square, London, W.C.* 1

In experiments designed to study possible interactions between learning with different sensory modalities, the results for the monkey were negative. Thus, with solid objects, differing in shape, no evidence was found of interaction (either facilitation or inhibition) between visual and tactile learning (Ettlinger, 1960). Similarly, with another group of monkeys there was no interaction between a rate discrimination learned visually or by hearing (Burton & Ettlinger, 1960). It was therefore considered of interest to establish whether cross-modal interaction might take place in man.

The method adopted was to examine the effect of auditory learning on visual learning. For this purpose normal adult subjects were taught to distinguish between pairs of auditory or visual temporal sequences produced by an apparatus previously described by Burton & Ettlinger (1960). Subjects were divided into three balanced groups. Those in control group I were taught to distinguish between two visual rhythms, and subsequently between the same rhythms presented for hearing. Those in control group II first learned to distinguish between two auditory frequencies and were then taught to make the distinction between the same visual rhythms as were the subjects in group I. Subjects in the experimental group were given alternating trails of auditory and visual rhythms. If auditory learning were to facilitate visual learning, the subjects in the experimental group might be expected to learn the visual rhythms more rapidly than the subjects in the two control groups. In other words, we have confined our analysis to performance on the visual rhythms.

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Under the conditions employed there was no indication that auditory learning had any effect on visual learning. Cross-modal interaction has therefore not been observed by us in man. The basis of interaction between differing sense modalities and the conditions under which it does or does not take place have yet to be established.

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Glucose consumption in man. A comparison of exogenous and endogenous insulin

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Man in the fasting state has a steady extracellular glucose concentration, the resultant of the processes which add new glucose molecules to it and those which remove them.

The fall in blood glucose concentration in the 90 min after disturbing the steady state with an intravenous injection of 25 g of glucose is the result of equilibration and utilization. Part of this fall behaves as a first-order reaction, and this has been used to calculate a rate constant, the 'Glucose Assimilation Coefficient', in normals and diabetics (Conard, Frankson, Bastenie, Kestens & Kovacs, 1953; Duncan, 1956; Heller, Kalant & Hoffman, 1958). The effect of insulin given intravenously with the glucose load on this coefficient has been considered a measure of 'Effective Insulin Activity'. We have observed that the coefficient is increased by a factor of times two or three (Heller *et al.* 1958).

In six out of eight cases with insulin-secreting tumours of the pancreas normal coefficients have been observed, both when the blood glucose level immediately before the glucose injection was low and when it was in the normal range. This suggests that the hypoglycaemia in these patients with insulinoma is not due to an increased rate of glucose utilization.

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The comparative efficiency of recognition in left and right visual fields

By MARIA WYKE*. *The Psychological Laboratory, Institute of Neurology, The National Hospital, Queen Square, London, W.C.* 1

Differences in the ability of visual recognition between the left and right fields of vision with brief exposures (100 msec) have been reported previously (Heron, 1957; Terrace, 1959). These differences were found in respect of the ability to recognize English words, but not with recognition of simple geometrical figures or of unfamiliar nonsense forms, when they were exposed successively in the fields of vision on the two sides of a centrally located fixation point. However, it has also been reported (Dallenbach, 1923) that when circular stimulus patches are exposed simultaneously in both half-fields of vision the left side is more 'attensive' (i.e. clearer or more vivid) for right-handed observers. In order to evaluate the differences in visual recognition when figures are presented successively or simultaneously in the right and left visual fields, tests of recognition of picture material were performed.

Outline drawings of familiar objects and animals were presented briefly (exposure times 10–40 msec), first successively in the left and right fields of vision. The same diagrams were then presented simultaneously in the homologous halves of the two visual fields. Finally, the same subjects were retested with the same diagrams at the same, and at shorter (5 msec), exposure times. The presentations of the picture material were made tachistoscopically.

The results obtained with eighteen adult normal subjects indicate that recognition of familiar objects when viewed for brief periods is more efficient on the right side of the binocular visual field than on the left. This applies whether the pictures are presented successively or simultaneously. The discrepancies in previous studies, and the possible explanation of the present results, are discussed.

The suggestion is made that there are differences in acuity between the two sides of the binocular field, acuity normally being better on the right.

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Weight changes in young men

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Body weight is one of the most frequently recorded parameters of man, but reliable records of daily weight changes for a period of a week or more are scanty. The subjects of this study were soldiers aged 17½–22 years, whose food intake and energy expenditure were measured for periods of 7 days. Body weight was recorded between 6·0 and 6.30 a.m., immediately after rising from bed and after the voiding of urine. An Avery balance was used, and the same team of two carried out all the measurements.

Changes in weight exceeding 0·5 kg between one day and the next were not infrequent. In order to follow the pattern of weight change, in any one individual, daily weighings were continued for periods up to 7 weeks. In over 1700 daily weighings, involving 64 subjects, in 30% of the occasions a weight change of 0·5 kg or more was recorded, and in one fifth of these the change was greater than 1·0 kg. Not only are such changes too large to be due to a calorie imbalance but similar changes were observed when the calorie balance was known, and could be shown to be quite inadequate to account for such a weight change.

No relationship has been found between energy expenditure and these large weight changes. Weight loss followed by recovery is commoner than an increase with a subsequent fall. In a subsequent study the results of measurement of the water intake and urine output gave support to the conclusion that water imbalance is responsible for these large and so far unpredictable changes.

The vascular responses of rats to posterior pituitary hormones after cerebral decortication

By Sybil Lloyd and Mary Pickford. *Physiology Department, University of Edinburgh*

Small doses of oxytocin (25–50 m-u.) have no effect on the blood pressure of normal dioestrous female rats, but a pressor response was invariably present after decerebration, pithing or sympathetic blockade, and the pressor response to vasopressin was increased (Lloyd & Pickford, 1960). In oestrous females, where pressor responses to both oxytocin and vasopressin normally occur, these were increased or unaltered by such procedures (Lloyd & Pickford, 1960). The present experiments were undertaken to determine whether the cerebral cortex was in any way concerned with the observed changes in response.

Blood pressure was recorded from a group of six anaesthetized female rats (for methods see Lloyd, 1959), and the responses to intravenous posterior pituitary hormones tested before and after removal of the cortex.

In all six rats after, but not before, decortication control injections of 0·3 ml. 0·9% NaCl solution caused some rise in blood pressure. In the three dioestrous females oxytocin had no effect on the blood pressure before decortication, and no greater effect than the control injections after decortication. In two oestrous rats and one in late pregnancy similar doses of oxytocin were pressor at all times (Fig. 1). The magnitude of the pressor response to vasopressin was never increased after decortication,

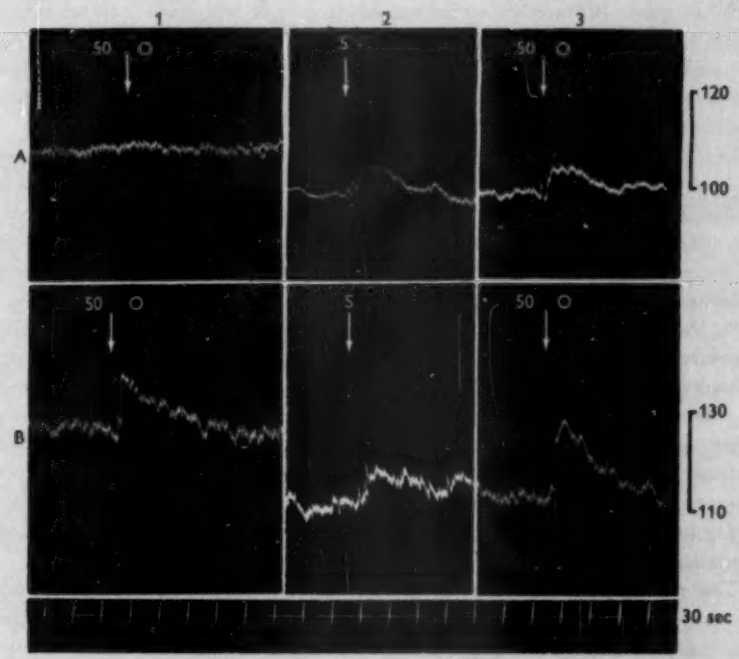

Fig. 1. Blood pressure responses of rats to intravenous oxytocin. A, dioestrous; B, oestrous. In both cases decortication between 1 and 2. In 1 and 3, 50 m-u. oxytocin; in 2, 0·3 ml. 0·9% NaCl solution.

but in most cases was slightly less than before operation. It is concluded that the cerebral cortex is not concerned in the alteration of the vascular responses to posterior pituitary hormones seen in rats after decerebration, pithing, or sympathetic blockade.

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The influence of guanethidine on the response of the human pulmonary circulation to hypoxia

By J. M. Bishop, P. Harris and N. Segel. *Department of Medicine, University of Birmingham, Queen Elizabeth Hospital, Birmingham*

The pulmonary arterial hypertension which occurs when hypoxic gas mixtures are breathed is generally thought to be due to vaso-constriction. It has been said (Judson, Hollander & Arrowood, 1954) that hypoxia does not cause pulmonary hypertension in people who have had an extensive sympathectomy, but others have found that sympathectomy does not affect the response (Fritts, Harris, Clauss, Odell & Cournand, 1958). Hexamethonium does not prevent the elevation in pulmonary arterial pressure (Judson, Hollander & Arrowood, 1954).

In the present investigation, the effects of breathing 12% oxygen on the pulmonary circulation of six normal human subjects have been compared before and after producing post-ganglionic sympathetic blockade with guanethidine. In each case the dose of guanethidine (10–27 mg intravenously) was sufficient to prevent the over-shoot in systemic arterial pressure which follows the Valsalva manoeuvre.

In the absence of guanethidine, hypoxia caused the pulmonary arterial pressure to rise from an average of 17 to 27 mm Hg. The pulmonary wedge pressure was unaffected. The cardiac output increased from an average of 4·0 to 4·8 l./min/m^2. Guanethidine itself had no consistent or substantial effect on the pulmonary arterial and wedge pressures or on the cardiac output. After guanethidine had been given, hypoxia caused the pulmonary arterial pressure to rise from an average of 18 to 29 mm/Hg. The pulmonary wedge pressure was unaffected. The cardiac output increased from an average of 3·8 to 4·5 l./min/m^2.

The results suggest: (1) that the effects of hypoxia on the human pulmonary circulation are not mediated by the sympathetic nerves; (2) that the sympathetic system plays no substantial role in governing the normal tone of the pulmonary vessels at rest.

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Studies on central action of tubocurarine

By W. Feldberg and K. Fleischhauer. *National Institute for Medical Research, Mill Hill, London, N.W. 7*

In cats anaesthetized with pentobarbitone, immobilized by flaxedil and artificially ventilated, tubocurarine (200–400 μg) injected into the lateral

cerebral ventricle produces: characteristic e.e.g. changes, pilo-erection, withdrawal of nictitating membranes, midriasis, and a rise in arterial blood pressure. Injected intracisternally it produces only a rise in blood pressure. All the other effects, therefore, do not require the tubocurarine to reach the subarachnoid space; they result from penetration into the walls of the cerebral ventricles. The fourth ventricle can be excluded because the effects occur on perfusion of tubocurarine 1/1000 from lateral ventricle to aqueduct. With this perfusion tubocurarine also produces a rise in blood pressure; thus there are two central sites from which tubocurarine produces pressor responses.

The characteristic e.e.g. changes recorded with epidural electrodes consist of surface-negative high-voltage spikes with frequencies first of about 6 and later up to 30/min. Initially each spike is a single sharp wave, later smaller waves appear on its descending limb. At times there are periods ('episodes') of fast synchronous high-voltage activity followed by a period of electrical silence. As described by Samson Wright (1955) these changes are more pronounced in the occipital than in the parietal and are not always present in the frontal lobes.

To elicit these e.e.g. changes the tubocurarine must act on structures reached from the lateral ventricle, because when perfusion, to either aqueduct or cisterna, was simultaneous from both lateral ventricles—from one with tubocurarine, from the other with artificial c.s.f.—the e.e.g. changes occurred only on the side perfused with tubocurarine. On the opposite side, either no abnormal activity was recorded, or at most it was slight. When the concentration of tubocurarine in the third ventricle was reduced by doubling the rate of flow from the side perfused with artificial c.s.f., the tubocurarine effects on the e.e.g. were not modified. This again suggests that they are brought about by the tubocurarine acting from the lateral and not from the third ventricle.

Pilo-erection occurred only after prolonged action of tubocurarine, indicating the need for its deeper penetration. At first there were rhythmic waves of pilo-erection on the tail, coinciding with the large spike discharges. Episodes were reflected by periods of maximal sustained pilo-erection on tail and back. There was also an association of the abnormal electrical activity with other autonomic responses. On the nictitating membranes, sudden small retractions coincided with spike discharges, and periods of maximal retraction with episodes. On the pupils, episodes were reflected by widening of their diameters with attenuation of oscillatory movements. Finally, on the blood pressure, after prolonged tubocurarine action, cessation of episodes coincided with transient falls.

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The contribution of the chemoreceptors to hyperpnoea induced by ammonium chloride

By N. Joels and E. Neil. *Department of Physiology, Middlesex Hospital Medical School, London, W. 1*

Anitschkov (1936) reported that the hyperpnoea caused by the administration of ammonium chloride to cats or dogs was little affected by chemoreceptor denervation. Winterstein & Gökhan (1953), on the other hand, claimed that carotid chemoreceptor denervation abolished the hyperpnoeic response of dogs to ammonium chloride.

We have re-investigated this problem in two sets of experiments performed on anaesthetized cats:

(1) The respiratory response to the intravenous injection of ammonium chloride (25 mg/kg body weight) was recorded before and after chemoreceptor denervation. In confirmation of the results of Anitschkov, chemoreceptor denervation had little effect upon the stimulation of breathing caused by ammonium chloride.

(2) Chemoreceptor impulse activity in thin twigs of the sinus nerve was recorded simultaneously with the electromyogram of the diaphragm or with the efferent impulse activity in thin slips of the recurrent laryngeal nerve. Diaphragmatic muscle potentials and the impulse salvoes in the recurrent laryngeal nerve were used as an indication of respiratory motor activity. The intravenous injection of ammonium chloride caused efferent respiratory responses which almost always preceded and invariably outlasted such moderate and transient increases of chemoreceptor discharge as did occur.

We conclude that such contribution that the chemoreceptors make to the hyperpnoea produced by ammonium chloride is of only secondary importance.

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The relationship between the onset of sweating and vasodilatation in the skin of the forearm during body heating

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When a cool subject is warmed the increase in forearm skin blood flow is due initially to a reduction in vasoconstrictor tone, followed by an active vasodilatation. The latter is the result of cholinergic nervous activity (Roddie, Shepherd & Whelan, 1957). It is not known whether this active vasodilatation can be wholly explained by the release of bradykinin

from the activated sweat glands (Fox & Hilton, 1958) or whether vasodilator fibres also act directly on the blood vessels. In the present experiments, the times of onset of sweating and of active vasodilatation have been carefully compared, to see whether or not active vasodilatation may occur before or coincidentally with sweating or after its onset.

Blood flow was measured in both forearms by venous occlusion plethysmography. Sweat was removed from the skin surface by a current of dry oxygen passing through a capsule on the volar aspect of the right forearm, absorbed in silica gel and weighed. The sweat was collected for consecutive periods of 2 min. The subjects, initially cool, were heated by immersing both feet in water at 43–44° C.

To define the time of onset of active vasodilatation the cutaneous nerves to the left forearm were blocked by infiltration with 3 % ethocaine hydrochloride containing 0·002 % adrenaline. Active vasodilatation in the right forearm was considered to begin when the blood flow in it exceeded that in the left. Using this criterion it was found that the onset of sweating invariably preceded active vasodilatation.

These findings provide no evidence for the existence of vasodilator nerves but do not exclude the possibility that such nerves may act on the blood vessels after sweating has commenced. They are compatible with the hypothesis that active vasodilatation is secondary to nervously induced sweat gland activity.

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Comparative aspects of cerebral haemodynamics

By B. Wyke. *Royal College of Surgeons of England, London, W.C.* 2

On the relationship between certain components of the human retinogram and of the occipital response to bright flashes

By W. A. Cobb and H. B. Morton. *Department of Applied Electrophysiology, The National Hospital, Queen Square, London, W.C.* 1

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